Hepatic Mitochondrial Renin-Angiotensin Systems

PhD Thesis

James R A Skipwork

MBBS, BSC(Hons), MRCS

Department of Surgery & Interventional Science,

University College London,

London, UK

Initial Statement

I, James R A Skipworth, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All methods and results have been reproduced and reported here faithfully and accurately, and in as much detail as possible. All experimentation was individually and personally performed by James R A Skipworth, except for electron microscopy imaging, which was performed by experienced electron microscopy technical staff at the Royal Free Hospital, UCL, London; and mass spectrometry, which was performed by experienced mass spectrometry staff at the Institute for Child Health (University College London, London) and the Taplin Mass Spectrometry unit (Harvard Medical School, USA).

Signed:……

Date:

……

Acknowledgements

I would like to express huge thanks to my supervisors: Professor Hugh Montgomery, Dr Gyorgy Szabadkai and Mr Steven Oldedamink for their seemingly endless font of advice and enthusiasm; and also Dr Jay Acharya, Dr Jutta Palmen, Dr Julie Sanders, Professor Michael Duchen, Professor Massimo Malago' and Professor Steve Humphries for their significant contributions, both practical and theoretical.

I remain indebted to the No Surrender Charitable Trust for their friendship and kind provision of funds, without which this project would not have been possible.

My friends and family were a constant source of encouragement and support, as they always have been, and for this I will always remain extremely thankful. My parents, in particular, have always provided a reference point to aspire to and have shaped me into the person I am today.

Finally, I would like to thank my wife, Bryony, and children, Jacob and Lucy, for their patience and forbearance during this long project, as well as their tolerance of my regular absence.

Abbreviations

ACE- Angiotensin converting enzyme; **ACE-I**- ACE inhibition; **ADP**- Adenosine diphosphate; **AGT**- Angiotensinogen; **Ang**- Angiotensin; **ANT**- ADP/ATP translocator; **ARB**- Angiotensin receptor blocker; **ATP**- Adenosine triphosphate; **ATR**- Angiotensin receptor; **AU**- Arbitrary units

BCA- Bicinchoninic acid; **BK**- Bradykinin; **BSA**- Bovine serum albumin

C- Cytoplasm; **cAMP**- Cyclic adenosine monophosphate; **CHF**- Congestive heart failure; **CM**- Crude mitochondria; **COX-2**- Cyclooxygenase-2; **CRP**- C-reactive protein

dCn- Delta correlation score; **DMEM**- Dulbecco's modified eagle's medium; **DNA**- Deoxyribonucleic acid; **DSS**- Disuccinmidyl suberate

ECM- extracellular matrix; **EM**- Electron microscope; **ER**- endoplasmic reticulum; **ESI**- Electrospray ionization; **ETC**- Electron transport chain

FAD- Flavin adenine dinucleotide; **FBS**- Foetal bovine serum; **FCCP**- Carbonilcyanide p-triflouromethoxyphenylhydrazone

GLUT- Glucose transporter; **grp75**- Glucose-regulated protein 75

H- Homogenate; **HSC**- Hepatic stellate cell; **HGF**- Hepatocyte growth factor

IP- Immunoprecipitation; **IP_2**- inositol 4,5-bisphosphate; **IP_3**- Inositol 1,4,5-trisphosphate

kDa- Kilodaltons

L- Lysosomes; **L-NAME**- N-nitro-L-arginine methyl ester

$\Delta\Psi m$- Mitochondrial membrane potential; **MALDI – TOF**- Matrix-assisted laser desorption ionisation – Time of flight; **MAM**- Mitochondrial associated membranes; **MAPK**- Mitogen-activated protein kinase; **MMP-2**- Matrix metalloproteinase 2; **mRAS**- Mitochondrial renin-angiotensin system; **mRNA**- Messenger RNA; **MS**- Microsomes; **MIF**- Macrophage migration inhibitory factor; **mW**- Molecular Weight

NAD- Nicotinamide adenine dinucleotide; **NADPH**- Nicotinamide adenine dinucleotide phosphate; **N**- Nucleus

O^2C- Change in Oxygen consumption; **OXA**- Oxidase assembly machinery

PBS- Phosphate buffered saline; **PLGS**- Protein Lynx Global Server; **PM**- Pure mitochondria; **PNGase-F**- Peptide N-glycosidase F; **PRR**- Prorenin receptor; **PVDF**- Polyvinylidene difluoride

RAS- Renin-angiotensin systems; **RAS-I**- RAS inhibition; **RNA**- Ribonucleic acid; **ROS**- Reactive oxygen species

SERCA- Sarco/endoplasmic reticulum Ca^{2+} ATPase; **SiRNA**- Small interfering RNA; **SS**- Size standards

TBS- Tris-buffered saline; **TGF**- Transforming Growth Factor; **TMRM**- Tetramethylrhodamine methyl ester; **tRNA**- Transfer RNA

UCP- Uncoupling protein

VDAC- Voltage-dependent anion-channel protein

XCorr- Cross correlation score

Abstract

Introduction: The circulating renin-angiotensin system (RAS) was originally described as a key endocrine regulator of intravascular homeostasis; however, the existence of a local (tissue) RAS has become increasingly reported in a variety of tissues including liver. RAS components have now also been detected in rat heart, brain and smooth muscle cell mitochondria as well as within intramitochondrial dense bodies of rat adrenal tissue. Further, reduced RAS levels have been associated with improved endurance performance and fatigue resistance in human skeletal muscle, suggesting that low RAS activity is associated with metabolic efficiency, potentially via RAS action upon, or within, mitochondria. However, such investigation has often relied heavily upon qualitative techniques (e.g. Western blotting, immunofluorescence and electron microscopy), which contain inherent limitations in that they completely rely upon the limited specificity of antibodies to demonstrate the existence of intra-mitochondrial RAS components.

Methods: The presence of RAS components within the mitochondria of rat hepatic tissue and liver cell-lines was investigated via sub-fractionation of rat liver tissue and cell-lines, followed by Western blotting, as well as via immunofluorescence and confocal microscopy, and electron microscopy. The mitochondrial effects of stimulating or antagonizing hepatic RAS were assessed via functional fluorescence microscopy (for assessment of NADH, calcium and mitochondrial membrane potential) and measurement of oxygen consumption within live cells of a liver cell-line.

Results: Western blotting, immunofluorescence and electron microscopy suggested the presence of RAS components within mitochondria; however, there was a lack of results consistency between techniques and the staining patterns were largely non-specific. Western blotting further demonstrated the presence of a prominent 55 kDa band, when immunostaining a mitochondrial fraction with (angiotensin-converting enzyme) ACE C-terminal antibody (usual size 180 kDa). This was further explored via isolation of the 55 kDa molecule and mass spectrometry to yield results consistent with non-specific staining only. Addition of RAS agonists or antagonists to live liver cell-lines demonstrated no consistent results, except at supra-physiological levels, where RAS antagonists improved oxygen consumption.

Conclusions: Such data suggest that the previous descriptions of RAS components within

mitochondria are likely to be secondary to methodological flaws, particularly the reliance upon single antibodies, which have subsequently been shown to have poor specificity. Thus, the effect of ang II on liver mitochondria is unlikely to be direct and any such action is likely to occur via one of several intracellular pathways, regulation of gene expression or mitochondrial biogenesis.

Contents

INTRODUCTION

I1. Renin-Angiotensin Systems

I1. a. Circulating Renin-Angiotensin Systems

The circulating (endocrine or systemic) renin-angiotensin system (RAS) was originally described as a key regulator of intravascular homeostasis, controlling extracellular fluid volume, blood pressure and absorption of electrolytes (Peach, 1977, Reid et al., 1978) (see Figure I1). In response to decreased afferent arteriolar pressure, decreased filtered sodium load in the nephron ultra-filtrate (from low plasma sodium concentrations or decreased glomerular filtration) or sympathetic nervous stimulation, the renal juxtaglomerular apparatus releases renin (Hackenthal et al., 1990). This aspartyl protease cleaves a leucine-leucine peptide bond in the hepatically-derived $\alpha 2$ globulin angiotensinogen to yield the non-pressor decapaptide angiotensin I (ang I) (Menard, 1993). Angiotensin-converting-enzyme (ACE) is a peptidyl dipeptidase which hydrolyses ang I to yield the pressor octapeptide angiotensin II (ang II) (Caldwell et al., 1976), whose effects are mediated through 2 specific human receptors (AT_1R and AT_2R) (Timmermans and Smith, 1994). The endocrine roles of the AT_2 receptor are less well defined (de Gasparo et al., 2000, Matsusaka and Ichikawa, 1997) than that of the AT_1R which, stimulated by ang II, mediates a hypertensive response through primary vasoconstriction and the release of aldosterone, the latter promoting sodium and water resorption from the distal convoluted tubule and collecting ducts (Matsusaka and Ichikawa, 1997). ACE is also a potent kininase, reducing the activity of bradykinin at its BK_1 and BK_2 receptors, capable of stimulating a variety of inflammatory and vascular responses, and ACE may also have similar regulatory actions upon other RAS components (Leeb-Lundberg et al., 2005, Campbell et al., 1994, Brown et al., 1998, Murphey et al., 2000, Margolius, 1996). Increasing circulating ACE activity therefore drives hypertensive responses (increased AT_1R receptor activation) and diminishes hypotensive responses (reduced vasodilation via BK_2 receptor activation). In these ways, circulating RAS plays a crucial role in the regulation of human blood pressure and salt and water balance (Kem and Brown, 1990).

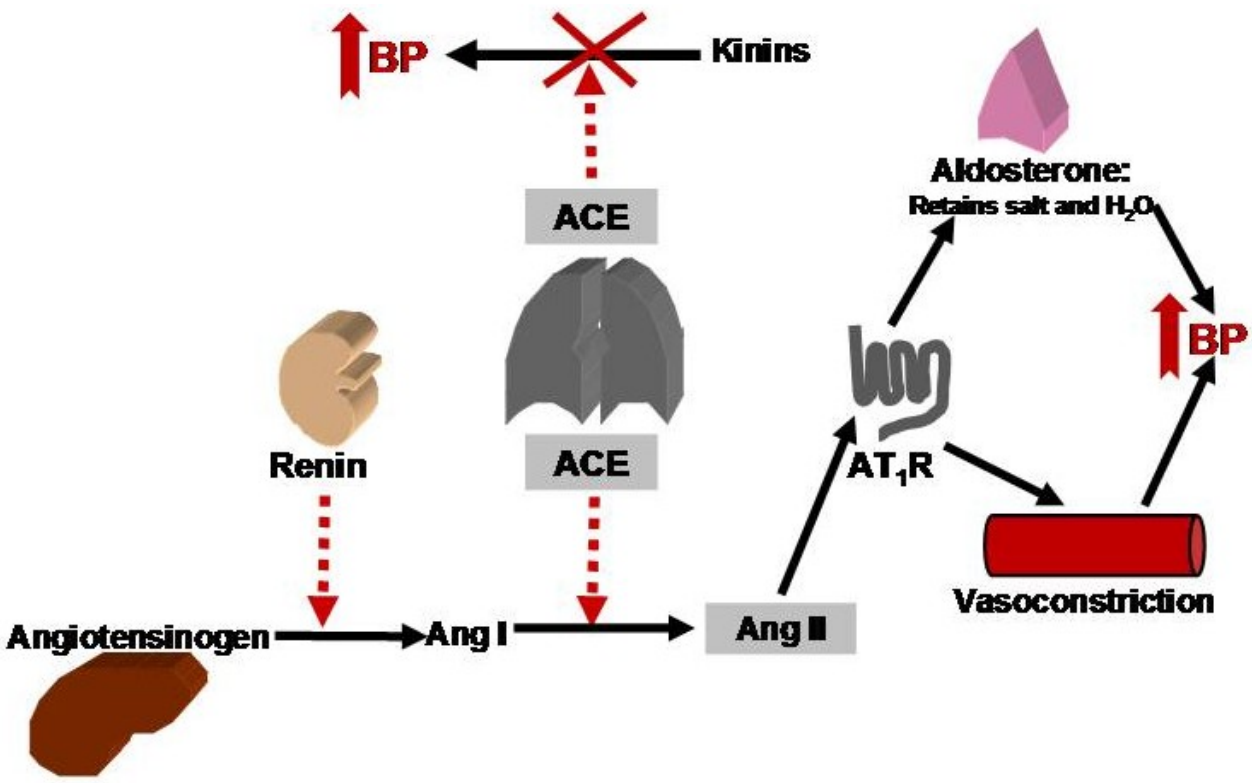

Figure I1. The traditional concept of a circulating, endocrine renin-angiotensin system, leading to hypertensive responses via angiotensin II action at the AT_1R (BP- Blood pressure; H_20- Water; ACE- Angiotensin converting enzyme; Ang- Angiotensin).

I1. b. Local Renin Angiotensin Systems: The RAS Concept Expanded

In the mid 1980s, imunohistochemical techniques demonstrated that proteins of the traditional RAS were present within cells and organs outside of that expected as part of its systemic, endocrine actions (Re, 1984). Such RAS presence has been termed 'local RAS' and have now been identified in tissues as diverse as the heart (Danser et al., 1995), kidney (Reams, 1992), vasculature (Paul et al., 2006), adipose tissue (Fowler et al., 2009), liver (Paizis et al., 2002), nervous tissue/brain (Reams, 1992), adrenals (van Kats et al., 2005), gonads/reproductive system (Leung and Sernia, 2003), the gastrointestinal system (Wong et al., 2007) and the pancreas (Lau et al., 2004, Paul et al., 2006). Local RAS play roles in signaling between different cell types (paracrine), cells of the same type (autocrine), or within a cell (intracrine role) (Rohrwasser et al., 1999). Such systems may be 'complete', where all RAS components are synthesised de novo, or 'depleted', being dependent for their

function on the uptake of some critical RAS components from the circulation (Campbell and Habener, 1986, Deschepper et al., 1986, Dzau, 1989). Such tissue RAS play myriad roles, including the regulation of cell growth, differentiation, apoptosis, and proliferation; metabolism and generation of reactive oxygen species and free radicals; tissue inflammation and fibrosis; local haemodynamics; and hormonal secretion (Leung and Ip, 2006, Ganong, 1995, Leung et al., 1999, Speth et al., 1999, Regulska et al., Paul et al., 2006, Hitomi et al., 2007, Touyz, 2005).

Local RAS demonstrate significantly more complexity than the traditional, circulating system, and include an alternative RAS pathway (ACE2-Ang(1-7)-Mas axis) for the cleavage of ang I (see Figure I2). ACE2, the principal processing enzyme of the alternative RAS pathway, is capable of cleaving the terminal leucine from ang I to generate ang II (1-9) (Donoghue et al., 2000), but it has 400-fold greater affinity for ang II itself (Vickers et al., 2002), from which it cleaves the terminal phenylalanine residue to synthesise ang (1-7), an angiotensin I heptapeptide (Tipnis et al., 2000, Turner et al., 2002). ACE2 has been identified in diverse *rodent* tissues including lung, adipose tissue (Gembardt et al., 2005), liver (Paizis et al., 2002), brain (Xia and Lazartigues, 2008) and pancreas (Tikellis et al., 2008), with confirmation in *human* heart, kidney, testis and small and large intestine (Donoghue et al., 2000, Tipnis et al., 2000), suggesting ubiquitous expression. Ang (1-7), the primary product of ACE2 action on ang II acts through the Mas receptor (Santos et al., 2003) and therefore has separate effects to the vasoconstrictor, pro-inflammatory and pro-oxidant effects mediated via ang II action on the AT_1 receptor. Mas receptor activation, by contrast, produces vasodilation via activation of bradykinin and nitric oxide (possibly via the Akt pathway (Sampaio et al., 2007, Toblli et al., 2008), prostaglandin release (Almeida et al., 2000), norepinephrine inhibition (Gironacci et al., 2004) and attenuation of inflammation and fibrosis via TGF and MIF (macrophage migration inhibitory factor) inhibition (Zhong et al., 2008, Grobe et al., 2007). ACE2 therefore negatively regulates the RAS and acts as an endogenous ACE inhibitor through alterations in formation of angiotensin II and angiotensin (1-7), thereby counter-balancing ACE action (see Figure I3). The alternative RAS pathway may act independently or in conjunction with ACE in the regulation of the local RAS, and may therefore be important in novel pathways of angiotensin metabolism and multiple pathogenic processes.

Within local RAS, a variety of ang I-processing enzymes exist in addition to ACE and ACE2, including chymase, chymotrypsin, tonin, aminopeptidase A, B and N, prolylendopeptidase, and neutral endopeptidase. Their actions yield a range of other biologically-active compounds. However, some of these enzymes will only become biologically active in pathological or abnormal conditions and the physiological relevance of such alternate ang-processing enzymes therefore requires further elucidation (Fyhrquist and Saijonmaa, 2008). Angiotensin II is degraded to ang III (heptapeptide angiotensin 2-8) by vascular and erythrocyte angiotensinases, and subsequently to hexapeptide ang IV (hexapeptide angiotensin 3-8), both of which have qualitatively similar circulatory effects to that of ang II, with ang III also acting via the AT_1R and ang IV having its own specific functional receptor (Fyhrquist and Saijonmaa, 2008).

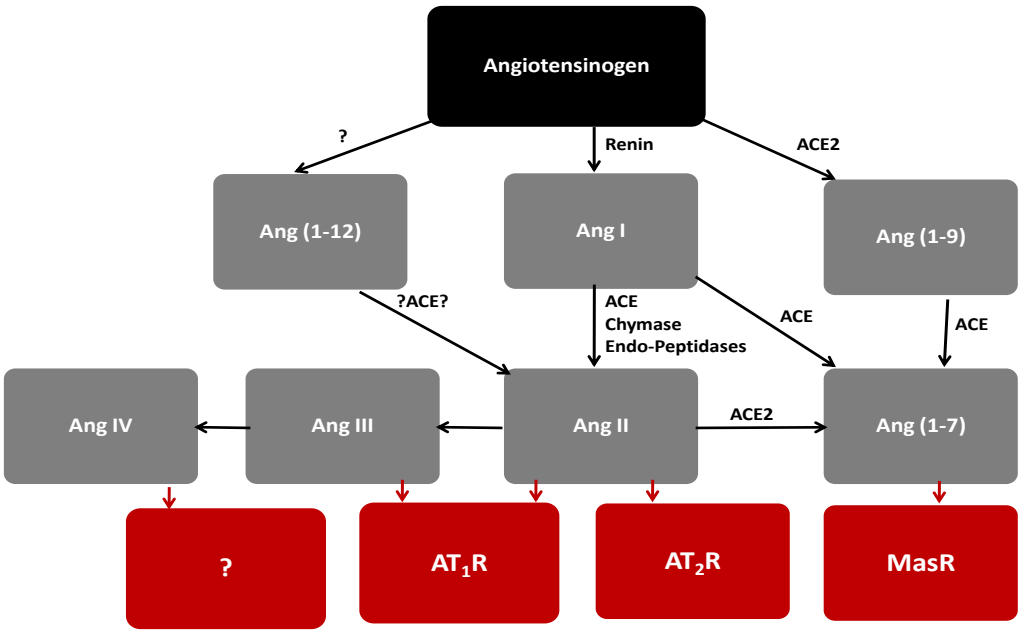

Figure I2. Local renin angiotensin systems: an expanded concept involving an alternative RAS processing pathway, via action of ACE2 (Ang- Angiotensin; AT_1R- Angiotensin II type 1 Receptor; AT_2R- Angiotensin II type 2 Receptor; MasR- Mas Receptor; ACE- Angiotensin converting Enzyme; ACE2- Angiotensin Converting Enzyme 2).

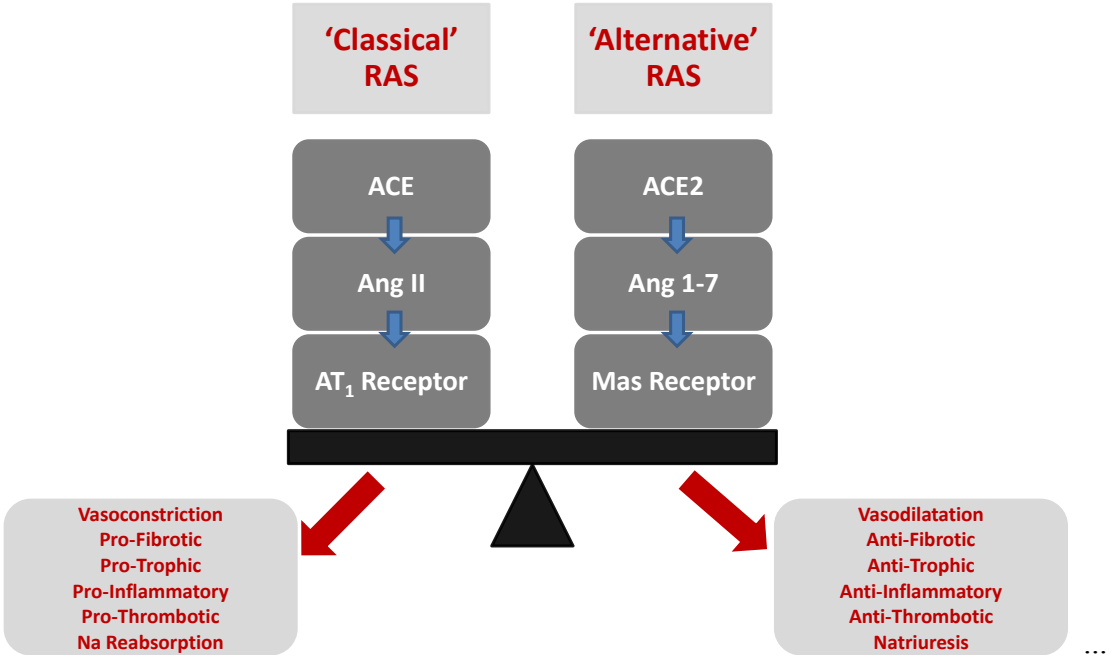

Figure I3. The various roles of the classical (endocrine) and alternative (novel) local renin-angiotensin systems are largely antagonistic (Ang- Angiotensin; AT_1R- Angiotensin II type 1 Receptor; MasR- Mas Receptor; ACE- Angiotensin converting Enzyme; ACE2- Angiotensin Converting Enzyme 2).

I1. c. RAS Components

I1. c. i. Renin

Renin is a highly-specific endopeptidase, secreted by granular cells of the renal juxtagomerular apparatus, whose primary endocrine function is to increase blood pressure and to this end renin hydrolyzes angiotensinogen to ang I. It is synthesized from renin precursor, a 406-amino acid peptide, which undergoes sequential proteolytic cleavage within the Golgi apparatus to an active, mature 340 amino acid (37 kDa) form (Imai et al., 1983, Pratt et al., 1983). Prorenin is glycosylated to various degrees, with higher levels of glycosylation affecting its cellular secretion and internalisation, via the mannose-6-phosphate receptor (Kumar et al., 2007). As part of the local RAS, renin can be synthesized intracellularly or taken up from the circulation, or both (e.g. cardiac myocytes) (Kumar et

al., 2007) and internalisation of non-glycosylated prorenin, via as yet unidentified receptors, is accompanied by similar increases in ang I and ang II, suggesting intracellular RAS synthesis (Kumar et al., 2007).

During condensation and packaging of the renin granules, a dipeptide near the carboxyl terminus is excised, converting renin into a two chain molecule combined by a disulphide bridge, which has less enzymatic activity than the single chain form (Pratt et al., 1983). Minor differences in amino acid composition, via variations in cleavage positions during processing, mean that multiple isoelectric forms of prorenin, one- and two-chain renin have been observed, with both one- and two-chain renin being secreted; whereas, prorenin is usually not (Pratt et al., 1983). The renin gene comprises 12 kb of DNA and contains 8 introns, producing multiple forms of mRNA to encode different renin isoforms (Hobart et al., 1984).

I1. c. ii. ACE

Angiotensin-converting enzyme (ACE) is a zinc-dependent dipeptidyl carboxypeptidase that belongs to the M2 family of zinc metallopeptidases and catalyses the hydrolysis of dipeptides from the carboxyl terminus of a wide variety of oligopeptides. The most celebrated physiological substrate for ACE is angiotensin I, which is converted into the potent vasopressor angiotensin II by removal of the C-terminal His-Leu; and the vasodilator bradykinin, which is inactivated by cleavage of the penultimate Pro-Phe bond. Other substrates of ACE include gonadotropin-releasing hormone (GnRH) or luteinising hormone-releasing hormone (LH-RH); substance P; b-neoendorphin1–9; and neurotensin, but the significance of these reactions *in vivo* remains uncertain.

An assessment of ACE's three-dimensional structure demonstrates that it is largely composed of alpha-helices, but also incorporates a zinc ion and two chloride ions. Thus, it bears little resemblance to carboxypeptidase A except in the presence of an active zinc-binding motif. This similarity extends to the active site, which consists of a deep, narrow channel that divides the molecule into two subdomains. At the superior aspect of the

molecule is an amino-terminal, which allows only small peptide substrates (2530 amino acids) access to the active site cleft, thus accounting for the inability of ACE to hydrolyse large, folded substrates.

ACE is expressed in two main forms derived from a single gene by alternative splicing. Both forms are type-1 membrane proteins (i.e. N-terminal regions are located extra-cellularly) but differ in their tissue expression and structure. The larger somatic ACE (sACE; 195kDa) is a ubiquitous, membrane-bound protein with two metalloproteinase domains (N- and C-terminal), each containing a canonical zinc-binding sequence motif (HExxH; His-Glu-x-x-His); and although both domains have protease activity (both are blocked by ACE-inhibitors) and are capable of converting ang I to ang II, the C-terminal domain is more important for blood pressure regulation. The smaller, secreted, testis-specific form of ACE (tACE; 90kDa) is composed of 27 helices (20 alpha-helices and seven 310-helices) and contains only the C-terminal metalloproteinase domain, together with the hydrophobic membrane-anchoring domain and a small N-terminal region that has multiple O-linked oligosaccharides; all of which packages into an ellipsoid shape with a central core channel (Sturrock et al., 2004). Both sACE and tACE are expressed on the cell surface, from where the biologically-active domains are released into the circulation by a cleavage-secretion process regulated by a small region in the C-terminal domain; and both forms convert inactive ang I to active ang II, as well as inactivating bradykinin (Chattopadhyay et al., 2008, Pang et al., 2001).

However, further, novel isoforms of ACE are also known to exist, with largely unknown functions and uncharacterized structures and synthetic pathways. Both 60 kDa and 130 kDa secretory isoforms exists, and an N-domain form also exists within urine in both 65 kDa and 90 kDa isoforms (Fernandes et al., 2008, Ronchi et al., 2005, Ronchi et al., 2007, Camargo de Andrade et al., 2006). Further, although ACE exists primarily as a membrane-bound form, a soluble form is present in plasma, amniotic fluid and other bodily fluids and is derived from the membrane-bound form by a post-translational proteolytic cleavage/shedding mechanism (Kumar et al., 2007).

I1. c. iii. ACE2

ACE2 (or ACE H) is an 805-amino acid protein initially isolated from a failing human heart ventricle and lymphoma cDNA libraries (Donoghue et al., 2000, Tipnis et al., 2000) and later confirmed as the receptor for the SARS coronavirus (Li et al., 2003), which shares 42% sequence homology with ACE (Donoghue et al., 2000), and which retains its catalytic domains (Donoghue et al., 2000, Tipnis et al., 2000). However, unlike ACE, and unique among mammalian carboxypeptidases, ACE2 contains a single HEXXH zinc-binding domain resulting in monocarboxypeptidase activity, although it does conserve other critical residues typical of ACE (Tipnis et al., 2000). ACE2 functions as a carboxypeptidase when acting upon ang I and II (Turner et al., 2002) and exists in both membrane-bound and soluble (generated by proteolytic cleavage of the ectodomain by tumour necrosis factor convertase, ADAM17 (Lambert et al., 2005)) forms (Donoghue et al., 2000), but neither hydrolyses or is inhibited by bradykinin, and is not sensitive to ACE-inhibitors despite sharing ACE's catalytic domain (Tipnis et al., 2000).

I1. c. iv. Angiotensins

Angiotensinogen (AGT) is a 452 amino acid α-2-globulin, of the serine protease inhibitor family (serpins), which is synthesised locally and then secreted extracellularly under the control of a signal sequence and the presence of glycosylation (Kumar et al., 2007). Multiple forms of angiotensinogen with varying degrees of glycosylation have been identified, suggesting complex intracellular distribution mechanisms (Kumar et al., 2007). AGT exists within the circulation as a reduced, unbridged form and an oxidised sulphydryl-bridged form (40:60), which preferentially interacts with renin (Zhou et al., 2010), cleaving the leu-val peptide bond and creating the decapeptide (des-Asp) ang I. AGT appears to have no independent function, existing solely as a precursor to biologically active ang II.

Ang II is formed by ACE-mediated removal of two C-terminal residues from ang I and its action via the AT_1R stimulates smooth muscle Gq proteins, activating vascular cell smooth muscle contraction by an IP3-dependent mechanism. Whilst circulating ang II has a half-life of approximately 30 seconds, its intracellular half-life may be as long as 15–30 minutes. Ang II can be further processed to fragments of its original size, including ang III (angiotensin heptapeptide 2-8) and ang IV (angiotensin hexapeptide 3-8), both of which

have significantly less pressor activity than ang II, although ang III has 100 % of the aldosterone-producing activity.

Ang III acts on the AT_1R to produce similar effects to ang II, including vasoconstriction and aldosterone release, and is generated from ang II predominantly by the action of aminopeptidase A; however, it is metabolically cleared five times faster than ang II (Fyhrquist and Saijonmaa, 2008).

Biologically active ang IV is generated from ang III by aminopeptidase M (predominantly), its receptor (AT_4R) being an insulin-regulated amino peptidase receptor (IRAP) found within a broad range of tissues, with particularly high expression in the brain (Kumar et al., 2007, Zhuo et al., 1998). The AT_4 receptor was originally described as a high-affinity binding site for ang IV, although the peptide LVV-hemorphin 7 has now also been demonstrated to be a biologically active AT_4R ligand (Chai et al., 2004). Agonist action of ang IV at the AT_4R causes improvements in memory and cognition, increased renal cortical blood flow and decreased Na^+ transport in renal proximal tubules, and may counteract AT_1R-mediated cardiovascular events such as injury in response to ischaemia and reperfusion, cardiac cell apoptosis and cardiomyocyte hypertrophy (Yang et al., Chai et al., 2004, Kerins et al., 1995).

Ang (1-7; angiotensin heptapeptide), the primary product of ACE2 action on ang II, acts through the Mas receptor (Santos et al., 2003) to produce antagonistic effects to ang II, and can also be generated by the action of other peptidases on ang II (Sampaio et al., 2007, Toblli et al., 2008, Almeida et al., 2000, Gironacci et al., 2004, Zhong et al., 2008, Grobe et al., 2007).

I1. c. v. RAS Receptors

AT_1R, AT_2R and the Mas Receptor are pharmacologically-distinct rhodopsin-like G-Protein Coupled Receptors (GPCR), integral membrane proteins with an extracellular amino

terminus, seven trans-membrane alpha-helical spanning regions with alternating intracellular and extracellular loops, and a cytosolic Carboxyl terminus. They activate a single or multiple G-proteins, each of which has 3 subunits (alpha, beta and gamma), of which the alpha subunit dissociates upon activation, rendering it active to affect intracellular signalling peptides or target functional proteins directly. Upon binding to ang II, the AT_1R translocates to the nucleus to affect transcription directly and resulting in reduced plasma membrane receptor density (Cook et al., 2006, Cook et al., 2004, Lee et al., 2004, Bkaily et al., 2003); however, significantly less is known of the function of the AT_2R, although its levels appear to be significantly higher in the foetus than in the adult.

Multiple specific antagonists have been identified for most RAS receptors including Losartan and PD123319, which are specific, competitive antagonists of the AT_1R and AT_2R, respectively.

I1. d. Specific Tissue RAS

I1. d. i. Hepatic RAS

All the components of the classical RAS (renin, angiotensinogen, ACE, ang II, AT_1R and AT_2R) have been identified in rat and human liver, where they have been implicated in the pathophysiology of various hepatic conditions, including chronic liver disease and fibrosis (Paizis et al., 2002, Paizis et al., 2005). Hepatic RAS activation and upregulation has been demonstrated following liver injury (from a number of various pathological insults), and RAS components have been shown to subsequently localize to areas of active fibrosis formation, where they can be identified in activated, but not quiescent, hepatic stellate cells (HSCs- crucial in the formation of liver fibrosis) (Bataller et al., 2003b, Bataller and Brenner, 2005, Friedman, 2008). Within healthy liver, quiescent HSCs express very low levels of RAS components and are unable to secrete ang II (Bataller et al., 2003b); however, following injury to hepatic tissue, HSCs become activated, increasing their expression of RAS components and acquiring the ability to synthesise ang II, which further stimulates activation and proliferation of HSCs via the AT_1R (Bataller et al., 2003b, Bataller et al., 2000), possibly through an oxidative stress and ROS generation-related mechanism (via

NADPH Oxidase and a JNK (c-\Jun N-terminal kinase)/ERK (extracellular-signal-regulated-kinase) transduction pathway) (Bataller et al., 2003a); and may also promote activation and differentiation of HSCs into myofibroblasts (Bataller and Brenner, 2005). Addition of ang II to hepatic tissue increases collagen expression and HSC secretion of cytokines, such as transforming growth factor-β1 (TGF-β1), effects which are attenuated by NADPH oxidase inhibition (Bataller et al., 2003c); whereas, systemic ang II infusion induces liver fibrosis and AT_1R antagonism or gene deletion attenuates liver fibrosis in rat models of hepatic injury (Bataller et al., 2000, Hirose et al., 2007, Kim et al., 2008, Ibanez et al., 2007, Ueki et al., 2006, Yang et al., 2005, Tuncer et al., 2003, Bataller et al., 2005). In keeping with this, a number of prospective human studies have shown improvements in liver fibrosis (secondary to viral hepatitis or NASH (non-alcoholic steato-hepatitis)) via treatment with competitive ACE inhibitors (e.g. Perindopril, Ramipril, Lisinopril) or AT_1R blockers (e.g. Losartan), further suggesting that ang II significantly contributes to the development of fibrosis (Colmenero et al., 2009, Debernardi-Venon et al., 2007, Sookoian et al., 2005, Terui et al., 2002, Yoshiji et al., 2005, Yoshiji et al., 2006, Yokohama et al., 2006). Further, the activation of HSCs by ang II promotes contraction of the hepatic sinusoidal vascular bed, contributing to the pathogenesis of portal hypertension in cirrhotic livers by increasing hepatic vascular resistance (Bataller et al., 2000, Rockey, 1997, Kaneda et al., 1998, Bhathal and Grossman, 1985).

In keeping, ACE expression and activity is increased in cirrhotic livers, stimulating local synthesis of ang II, within a circulatory environment (in cirrhotic patients) that has been shown to already be more responsive to ang II (Herath et al., 2009, Paizis et al., 2002). Further RAS effects within hepatocytes can be seen upon mitotic and proliferation rates (Cook et al., 2001): in rat hepatoma cells transfected with mutated angiotensinogen cDNA (signal sequence-encoding region removed, to differentiate the relative effects of nuclear and cell surface ang II receptors), a pro-mitotic/proliferation effect (caused by autocrine/paracrine stimulation of platelet-derived growth factor) can be blocked by Losartan (competitive AT_1R antagonist). The same cells were also found to produce an alternative renin transcript, renin 1A, which stayed intracellularly and lacked a signal sequence. There is also evidence for a counter-balancing effect of the novel RAS pathway in the prevention of liver fibrosis and ang IV analogues have been shown to bind directly to hepatocyte growth factor (HGF), where they antagonize its ability to form functional

dimers, thus impairing HGF-dependent signaling, proliferation and scattering in multiple cell-types (Kawas et al., 2012).

I1. d. ii. RAS in other tissues

RAS expression has also been discovered in other tissues such as heart (Pieruzzi et al., 1995), kidney (Pieruzzi et al., 1995, Neubauer et al., 2009), vasculature (Neubauer et al., 2009), lung (Pieruzzi et al., 1995), brain (Lenkei et al., 1997), pancreas (Leung et al., 1997, Leung et al., 1999, Leung, 2007, Leung and Carlsson, 2001), small bowel and colon (Paul et al., 2006), and adrenal tissue (Nostramo et al., 2012). Thus, local tissue RAS are expressed in a variety of tissues and organ systems, where they play a role in multiple physiological and pathophysiological processes. The dual role of RAS, in that both its endocrine and paracrine systems act simultaneously, means that the system is extremely complex and its underlying mechanisms difficult to clearly elucidate. One of the major difficulties stems from the limitations of pharmacology in differentiating between RAS products synthesised locally and those generated systemically; however, it remains clear that tissue RAS play a crucial role in the functional regulation of the organ system in which they are produced. Thus, drugs including ACE-inhibitors, AT_1 antagonists and renin inhibitors offer extraordinary therapeutic potential.

I1.d. iii. Intracellular RAS expression

Extracellular (endocrine and paracrine) RAS executes the classical functions of ang II via activation of cell surface G-protein coupled angiotensin receptors, AT_1R and AT_2R (Campbell, 1987, Carey and Siragy, 2003, de Gasparo et al., 2000, Mehta and Griendling, 2007, Touyz and Schiffrin, 2000, Timmermans et al., 1993). However, such actions of ang II cannot explain the myriad roles of the RAS, or its antagonism, in both physiology and pathophysiology, leading various researchers to further investigate a putative intracellular RAS. In particular, ang II addition results in longer-term (hours, days, weeks) effects upon growth, mitogenesis and cellular proliferation, which are independent from the classical systemic effects (mediated via cell-surface receptors) occurring within seconds or minutes; and the lipophilicity of some RAS antagonists renders them less capable of crossing the cell membrane, resulting in differential effects due to preferential actions at extracellular or intracellular RAS receptors. Some antagonists are therefore less effective at preventing the cardiac and renal complications for which they have been prescribed, suggesting that the pathogenesis of such disease derives from intracellular RAS, and further highlighting the

necessity of intracrine RAS investigation. For such investigation, experimentation has focused predominantly upon renal and adrenal tissue as these tissues not only contain all RAS components but their presence has been measured at high tissue concentrations (Ingert et al., 2002).

The classical form of renin is a 339 to 343 secretory amino acid peptide formed by proteolytic cleavage of the prorenin N-terminus. The kidney is the main site of secreted active renin and prorenin, although other tissues also secrete prorenin into the circulation (Sealey et al., 1986), where it acts as the circulatory precursor for active renin but can also be taken up into various tissues in its precursor form. Intracellularly, renin appears to be targeted to lysosomes, the regulated secretory pathway or alternate intracellular sites, with specific sorting being determined by the cell type and the precise expression and structure of the renin transcript in question. Within renal tissue, renin and prorenin are primarily located within cells of the juxtaglomerular apparatus, afferent arterioles of smooth muscle and interlobular arterioles (Darby et al., 1985, Song et al., 1992, Taugner et al., 1981, Zhuo et al., 1996), where renin is a secretory protein that increases vascular tone, glomerular filtration pressure and sodium reabsorption via a negative feedback mechanism (Hackenthal et al., 1990). Here, renin is located in storage granules that demonstrate calcium-induced exocytosis, and are thought to be modified lysosomes as they contain lysosomal enzymes and phagocytic properties (Hackenthal et al., 1990, Mullins et al., 1982, Panthier et al., 1982, Sharp et al., 1996, Clark et al., 1997, Mullins et al., 1990, Peters et al., 1993). A prorenin receptor (PRR; a 350 amino acid peptide with a single transmembrane domain) has also been discovered within glomerular mesangial cells, distal nephrons and collecting ducts (Advani et al., 2009, Nguyen et al., 1996, Sihn et al., 2010) (as well as being expressed in widely different tissues) to which both renin and prorenin are capable of binding. The PRR induces multiple physiological and pathophysiological effects such as activating ERK 1/2 MAP (extracellular signal regulated kinase 1/2 mitogen-activated-protein) kinase intracellular signaling pathways, p38 MAP kinase, COX-2 (cyclooxygenase-2) and fibronectin (Nguyen, 2010, Nguyen, 2011, Sihn et al., 2010, Nguyen and Contrepas, 2008). However, it remains unclear what the overall physiological role of the PRR is and whether it is preferentially physiologically activated by renin or prorenin (Nabi et al., 2006, Nguyen et al., 2002). There is some evidence that prorenin is taken up by various tissues where it may aid in generation of intracellular ang II either via an intracellular route (Nguyen et al., 1996,

Prescott et al., 2002) or via prorenin activation at the cell surface, as well as potentially mediating intracellular actions of renin and prorenin (Danser, 2009, Sihn et al., Advani et al., 2009, Nguyen et al., 2002, Saris et al., 2006).

However, within the adrenal gland, alternative renin transcripts have been identified which encode for a non-secretory form of renin and adrenocortical cells are capable of storing renin in its active form without requiring glycosylation prior to storage, as in renal tissue (Clausmeyer et al., 1999, Lee-Kirsch et al., 1999). Here, circulating ang II induces both adrenal aldosterone and local renin synthesis; thus, producing a positive feedback loop to further increase local ang II generation. Adrenal tissue is therefore not reliant upon the uptake of ang II from the circulation for the production of aldosterone and the non-desirable systemic results of increased ang II, such as the promotion of fibrosis, hypertrophy and proliferation are contained (Hollenberg et al., 1974, Williams and Hollenberg, 1991, Peters and Ganten, 1998).

In the heart however, renin is not produced intracellularly under normal conditions (or is produced in extremely low amounts) and cardiomyocytes therefore predominantly rely upon mannose-6-phosphate receptor mediated endocytosis for uptake of renin from the circulation to lysosomal-like storage granules (reliant upon glycosylation, similar to renal tissue) (Ekker et al., 1989, Danser et al., 1994, van Kesteren et al., 1997, Saris et al., 2001). An independent pathway also internalises prorenin (following which it is subsequently cleaved to active renin), with unglycosylated (but not glycosylated) uptake leading to increased intracellular ang II (presumably due to intracellular ang II generation) (Peters et al., 2002). Thus, rats expressing high circulating prorenin result in high intracellular ang II and cardiac damage, suggesting that prorenin uptake may be more important than active renin uptake in cardiac tissue (Peters et al., 2002).

Most circulating angiotensinogen is synthesized and secreted by the liver but some is also generated within the kidneys (Kobori et al., 2006, Darby and Sernia, 1995). Angiotensinogen has also been located in perinuclear regions of glial cells (Sherrod et al., 2005); although whether it is synthesized locally at extra-hepatic and extra-renal sites, or taken up from the circulation, remains unknown. Agt produced locally in proximal tubule

cells is both secreted (into the tubular lumen) and contained intracellularly, where it leads to ang II formation (Lantelme et al., 2002, Navar et al., 2001, Rohrwasser et al., 1999).

ACE has been localized within multiple tissues including kidney, although intracellular expression levels in humans appear to be much lower than that of many experimental animals, including rats and dogs (Metzger et al., 1999). This may explain the significantly lower rates of ang I to ang II conversion in human renal tissue (approx. 10 % of arterially delivered ang I is converted to ang II) (Danser et al., 1998, Rosivall and Navar, 1983) and may imply that alternate (non-ACE) angiotensin processing enzymes are more important for the formation of intracellular ang II. In keeping, cathepsin D catalyzes the formation of AGT to ang I in rat vascular smooth muscle cells (Lavrentyev et al., 2007, Lavrentyev and Malik, 2009); and the content of intracellular ang II increases in diabetic rats with greater oxidative stress, via a non-ACE-dependent pathway (Singh et al., 2007, Singh et al., 2008). However, ACE activity remains important in renal tissue, where it is crucial in the maintenance of steady state ang II levels (Modrall et al., Ingelfinger et al., 1999, Kobori et al., 2001a, Kobori et al., 2001b, Kobori et al., 2002).

In proximal tubule cells, high-density, high-affinity AT_1 receptors are localised on both basal and apical membranes, where binding of circulating and paracrine ang II leads to internalization of the ligand-receptor complex (Brown et al., 2009, Hunyady, 1999, Li and Zhuo, 2008a, Thekkumkara and Linas, 2002, Brown and Douglas, 1982, Douglas, 1987, Dulin et al., 1994, Cogan, 1990, Harris and Navar, 1985, Houillier et al., 1996, Von Thun et al., 1994, Wang and Chan, 1991, Zhuo et al., 1992, Imig et al., 1999, Shao et al., 2009, van Kats et al., 2001, Zhuo et al., 2002, Zhuo and Li, 2007, Zou et al., 1998). It has been postulated that this internalization process helped to desensitise the cell to ongoing ang II stimulation by removing an activated receptor from the cell surface and translocating it to the cell interior where it is subsequently recycled, whilst ang II is delivered either to the endosomal or lysosomal pathway (Ferguson, 2001, Gonzalez-Gaitan, 2003, Hunyady et al., 2000, Zhang et al., 1997, Hein et al., 1997). In keeping, AT_1R blockade or deletion significantly reduces intra-renal ang II content, which is restored by ang II administration, suggesting that, in renal tissue at least, ang II is taken up from outside the cell or leads to stimulation of intracellular generation, and internalisation of the receptor-ligand complex

may aid in the regulation of this pathway (Campbell, 1996, Cervenka et al., 2008, Li et al., 2007, Li and Zhuo, 2007, Sadjadi et al., 2005). Once within the cell, it is not clear how ang II escapes the lysosomal or endosomal pathway to bind intracellular receptors; although endosomal sorting may provide a delayed intracellular release mechanism to protect from immediate ang II metabolism (Imig et al., 1999) or a pathway to aid in regulation of continuous ang II signaling (Murphy et al., 2009).

The precise mechanisms underlying ang II-receptor complex endocytosis have not been clearly elucidated and in non-renal cell types, such as vascular smooth muscle cells, a clathrin-coated pit-dependent pathway would appear to be the crucial mechanism for ang II-receptor uptake (Anborgh et al., 2000, Ferguson, 2001, Rajagopal et al., 2006, Thomas et al., 1996) and in other cell types non-canonical, alternate pathways may also be important (Hunyady et al., 2000, Rajagopal et al., 2006, Li et al., 2006, Zhuo and Li, 2007, Zhuo et al., 2006a, Li et al., 2009). Following internalization, ang II pathways and trafficking mechanisms remain largely unknown. Internalised ang II could have a number of functions, including recycling and secretion for extracellular action, migrating to the nucleus to exert transcriptional effects or undergoing processing to enable it to act intracellularly, potentially via stimulation of the inositol 1,4,5 triphosphate pathway (Chen et al., 2000). A nuclear targeting and activation sequence was recently discovered in the AT_1R (Morinelli et al., 2007) and AT_1 receptors have been discovered in peri-nuclear and nuclear regions of HEK 293 (human embryonic kidney) cells, cardiomyocytes, vascular smooth muscle cells and hepatocytes; although, whether these represent typical or internalized cell-surface receptors remains unknown (Chen et al., 2000, 2007, Licea et al., 2002, Cook et al., 2006, Cook et al., 2007). Ang II action, via nuclear AT_1 receptors, results in increased transcription of TGF-1 (tumour growth factor-1) and MCP-1 (monocyte chemoattractant protein-1), as well as increased nitric oxide and superoxide production, in isolated rat renal cortical cells (Gwathmey et al., 2009, Gwathmey et al., Li and Zhuo, 2008b); whereas, micro-injection of ang II (one method of directly investigating intracellular effects) leads to intracellular and nuclear calcium increases in a variety of cell-types including proximal tubule and vascular smooth muscle cells (Haller et al., 1996, Haller et al., 1999, De Mello, 1998, De Mello, 2006, Zhuo, 200). In keeping, micro-injection of Losartan prevents calcium responses secondary to micro-injected ang II, but not those secondary to extracellular ang II, suggesting that direct intracellular effects are occurring (Zhuo et al., 2006b).

I2. A Metabolic Role for RAS

I2. a. RAS and Performance

The powerful benefits of ACE inhibition (ACE-I) in the treatment of heart failure have yet to be fully explained. Similarly, the mechanisms by which ACE inhibition reduces long-term cardiovascular risk are incompletely understood. In both cases, a role for altered cellular metabolism has been postulated. Renin-angiotensin system activity is associated with variations in human physical performance: the presence (Insertion, I allele) rather than the absence (Deletion, D allele) of a 287 base pair fragment in the ACE gene is associated with lower circulating (Rigat et al., 1990) and tissue (Danser et al., 1995, Costerousse et al., 1993) ACE activity, which in turn is associated with fatigue resistance (Montgomery et al., 1998) and endurance performance (Myerson et al., 1999, Tsianos et al., 2005). Amongst British Olympic-standard runners, I allele frequency increases with distance run (I allele proportion 0.35, 0.53 and 0.62 amongst those running ≤ 200 m, 400-3000 m and ≥ 5000 m respectively ($p = 0.009$)) (Myerson et al., 1999). Amongst young male army recruits undergoing 12 weeks of military physical training, duration of standardised repetitive loaded (15 kg) elbow flexion improved by 79.4 ($\pm$25.2), 24.7 ($\pm$8.8), and 7.1 ($\pm$14.9) seconds for those of II, ID and DD genotype respectively ($p<0.001$ for II vs. DD) (Montgomery et al., 1998). Such improvements in endurance performance/fatigue-resistance are thought to represent improved muscle metabolic efficiency, suggesting that low ACE activity (marked by the I allele) is associated with enhanced muscle metabolic efficiency.

Such associations may in part be mediated through altered cellular metabolism: low ACE genotypes are associated with a relative conservation of fat stores during high-intensity exercise training (Montgomery et al., 1998), greater training-related gains in 'delta efficiency' (the ratio of the change in muscle work performed/min to the change in energy expended/min (measured in a regression equation via assessment of rate of oxygen uptake and respiratory exchange ratio, while performing on a cycle ergometer) (Williams et al., 2004)) and reductions in oxygen consumption per unit of external work (Woods et al., 2002), and especially with successful performance in hypoxic environments (Montgomery et al., 1998, Tsianos et al., 2005). The I allele has been associated with increased maximum

altitude achieved (a form of extreme *hypoxic* endurance exercise) during ascent among elite high altitude mountaineers (8559 +/- 565 m for IIs, 8107 +/- 653 m for IDs and 8079 +/- 947 m for DDs (p = 0.007)) (Thompson et al., 2007); and similarly amongst ascent to the summit of Mont Blanc (100% of II, 94.9% of ID, 87.7% of DD individuals; P=0.048) (Tsianos et al., 2005), effects which may in part depend upon regulation of BK_2 receptor activity (Williams et al., 2004). The I-allele has similarly been associated with successful performance in ascents to 4800 m and beyond 8000 m (Thompson et al., 2007, Tsianos et al., 2006, Tsianos et al., 2005).

In keeping, pharmacological ACE inhibition protects the heart from subsequent ischaemic injury (Marktanner et al., 2006), whilst genetic elevation of ACE activity negates such preconditioning effects (Messadi et al., 2010). Combined ACE inhibition and AT_1R antagonism reduce renal oxygen consumption related to sodium transport (Deng et al., 2009). Meanwhile, ang II infusion increases oxygen consumption in the isolated perfused hindlimb (Colquhoun et al., 1988) and in liver (Matsumura et al., 1992), effects which may contribute (Cassis et al., 2002) to the (pressor-independent) muscle wasting seen with systemic ang II infusion (Brink et al., 2001).

These data support a role for lower ACE activity interacting with physical training in the regulation of metabolic efficiency. They suggest *increased* RAS activity to be associated with *reduced* metabolic efficiency. Conversely, the benefits of ACE inhibition in patients with CHF (congestive heart failure) seem mediated though peripheral (skeletal muscle) metabolic effects rather than through central (cardio-respiratory) effects (Jondeau et al., 1997). Forearm blood flow (measured via plethysmograph) is lower in CHF than control subjects (whilst performing the same load during dynamic handgrip exercise), both during rest and exercise, and is compensated for by increased oxygen extraction (thus maintaining forearm oxygen consumption); however, captopril (ACE-I) use increased forearm oxygen extraction and blood flow, thus increasing efficiency of oxygen utilization (Imaizumi et al., 1990). In rats with CHF, muscle ATP and creatine phosphate levels decline more quickly with exercise than in controls, and lactate levels rise faster. Six weeks of ACE inhibition reverses these effects, suggesting that ACE inhibition may restore skeletal muscle metabolic efficiency (Yamaguchi et al., 1999); similarly, 10 weeks of ACE inhibition in rats leads to

an increase in functional response to endurance training (Habouzit et al., 2009). Such effects on metabolic efficiency may account for the dramatic beneficial effects of treatment with ACE inhibitors in patients with CHF (Jondeau et al., 1997).

I2. b. RAS and Cachexia

The mechanisms underlying the development of cachexia remain unclear, although ang II levels appear to be associated in a causal fashion, acting via anorexigenic and catabolic effects (Brink et al., 2001). While ang II infusions in rats led to transient initial decreases in oxygen consumption; significant reductions in weight and food intake at 28 days were associated with a rebound increase in oxygen consumption, potentially contributing to sustained reductions in body weight (Cassis et al., 2002). Ang II addition has been shown to result in decreased protein synthesis and increased protein degradation via stimulation of the ubiquitin-proteosome pathway (a pathway of cytosolic protein degradation involving ubiquitin, a 7.5kDa, 76-amino acid ATP-dependent protease that is found ubiquitously throughout the cell and proteosomes, specific protein-degrading organelles) and ang II-mediated activation of protein kinase C (Sanders et al., 2005). Further, gastrointestinal and lung cancer patients with an ACE D allele exhibit lower total fat mass, percent body fat and lean body mass when compared to an II genotype patient group (Vigano et al., 2009).

I3. RAS & Mitochondria

I3. a. Mitochondrial Structure & Function

Mitochondria are membrane-enclosed organelles found within the cells of most eukaryotes, and which are responsible for the production of adenosine triphosphate (ATP), the high-energy molecule utilized as energy in most cellular processes. Mitochondria range in size from 0.5-10 μm in diameter and the number of mitochondria in a cell varies widely by organism and tissue type: many cells have only a single mitochondrion, whereas others can contain several thousand. Hepatocytes, in particular, contain 1000-2000 mitochondria per cell, comprising up to 20 % of cell volume. Mitochondria are usually located between myofibrils, often forming a branched network with the cytoskeleton, which aids in maintenance of mitochondrial shape and can affect mitochondrial function.

I3. a. i. Outer Mitochondrial Membrane

The outer membrane encloses the entire organelle, which is separated by an intermembrane space from an inner mitochondrial membrane, which, in turn, bounds the mitochondrial matrix. Both outer and inner membranes are composed of phospholipid bilayers and proteins, although the outer membrane has a protein-to-phospholipid ratio similar to eukaryotic plasma membrane (approximately 1 : 1 by weight). The outer membrane also contains large numbers of integral proteins (porins) forming channels that allow small molecules to freely diffuse across the membrane. Larger proteins can only actively enter the mitochondrion via the binding of an N-terminus signalling sequence to a translocase within the outer membrane. The outer membrane is also capable of associating with the membrane of the endoplasmic reticulum, forming a structure called MAM (mitochondria-associated-membranes) that is crucial in ER-mitochondria calcium signalling. Outer membrane disruption allows proteins within the intermembrane space to leak into the cytosol, subsequently resulting in cell death.

I3. a. ii. Inner Mitochondrial Membrane

The inner membrane contains over 100 different polypeptides and has a high protein-to-phospholipid ratio (over 3:1 by weight), and therefore contains approximately 20% of all mitochondrial protein. Despite this, it contains no porins and is rich in cardiolipin, rendering it highly impermeable. Thus, almost all ions and molecules require specific membrane transporters to enter or exit the matrix. The inner mitochondrial membrane contains numerous invaginations, called cristae, which expand the surface area of the inner mitochondrial membrane to up to five times greater than that of the outer membrane in hepatic cells and even more in skeletal muscle cells, thus enhancing its ability to produce ATP. The inner membrane contains the four complexes of the electron transport chain (ETC; complex I-IV) and the cristae are studded with oxysomes (F1 or elementary particles), which contain ATP synthase for ATP production. The inner membrane also has an electrochemical gradient across it formed by the action of the ETC enzymes.

I3. a. iii. Intermembrane Space

The intermembrane space is bound by the outer and inner membranes and has similar concentrations of small molecules to cytoplasm (due to the free permeability of the outer membrane), although concentrations of large molecules remain different (due to the fact that specific membrane transporter proteins are required for larger molecules to cross the outer membrane). Cytochrome c, a small haem protein crucial to mitochondrial function, is specifically located to the intermembrane space, via its anchoring with cardiolipin, where it carries electrons from complex III to IV, as well as playing a role in initiation of apoptosis.

I3. a. iv. Mitochondrial Matrix

The matrix contains a highly concentrated mixture of enzymes responsible for oxidation of pyruvate and fatty acids, and the Krebs cycle (Citric Acid/ Tricarboxylic Acid), and contains approximately two thirds of mitochondrial protein, as well as ribosomes, RNA, and several copies of the mitochondrial genome.

I3. a. v. Mitochondrial Genome & Proteome

Mitochondria have an independent genome, although the precise nature of mitochondrial proteins varies depending upon the tissue and species that the mitochondrion is derived from. In humans, the mitochondrial genome is a circular DNA molecule of approximately 16 kilobases, encoding 615 distinct protein types, derived from 37 total genes (13 peptide genes). Thirteen genes encode for the subunits of respiratory complexes I, III, IV and V, 22 for mitochondrial transfer RNA (tRNA) and 2 for ribosomal RNA. The genes are highly conserved but may vary in location and each mitochondrion can contain up to ten copies of its DNA, which has a high proportion of coding DNA and an absence of introns and repeats. Mitochondrial genes are transcribed as multigenic transcripts, which are cleaved and polyadenylated to yield mature mRNAs.

Proteins not encoded by the mitochondrial genome must therefore enter from the cytoplasm, with small molecules entering by diffusion via porins, whilst others are actively transported. Human mitochondria are also capable of importing both messenger RNA (mRNA) (Ahmed and Fisher, 2009) and tRNA (Rubio et al., 2008). Diverse proteins (beyond those needed for the metabolic pathways described above) are also imported after binding to translocase of the outer mitochondrial membrane, and thence to distinct translocases of the inner mitochondrial membrane (Rehling et al., 2001). A few proteins are synthesized on matrix ribosomes and exported into the inner membrane by the oxidase assembly (OXA) machinery (Schmidt et al., 2010). As a result, whilst the mitochondrial genome encodes only 13 proteins, upwards of 1450 are identified in the human mitochondrial proteome (Human mitochondrial protein database, 2010), whose folding and assembly are maintained by a complex machinery which includes dedicated proteases and chaperones (Haynes and Ron, 2010).

I3. a. vi. Mitochondrial Function

Mitochondria are responsible for energy production via respiration (see Figure I4). Through glycolysis within the cytosol, glucose is converted to two pyruvate molecules, which are transported across the inner mitochondrial membrane and are subsequently oxidized and combined with coenzyme A to form carbon dioxide, acetyl-CoA and NADH by the pyruvate

dehydrogenase complex within the mitochondrial matrix. Acetyl-CoA is the primary substrate of the Krebs cycle, which acts largely within the mitochondrial matrix (apart from succinate dehydrogenase, which is bound to the inner mitochondrial membrane as part of complex II), each cycle yielding three reduced nicotinamide adenine dinucleotide molecules (NADH) and one reduced flavin adenine dinucleotide molecule ($FADH_2$), which act as a source of electrons for the electron transport chain (ETC), thus driving ATP synthesis via oxidative phosphorylation.

The ETC comprises four separate protein complexes within the inner membrane, complex I (NADH reductase), II (succinate dehydrogenase), III (cytochrome c reductase) and IV (cytochrome a). Redox energy from NADH and $FADH_2$ is transferred to oxygen via the passage of electrons down the chain from one complex to the next, each of which has a greater reduction potential than the last. At each step, the incremental release of energy is utilized to pump hydrogen ions across the inner mitochondrial membrane into the intermembrane space, creating a strong, electrical, chemi-osmotic gradient across the inner membrane as the proton concentration in the intermembrane space increases. The subsequent return of these hydrogen ions across the membrane and into the matrix drives the synthesis of ATP, by ATP synthase, from ADP and inorganic phosphate (oxidative phosphorylation), with hydrogen ions, electrons and oxygen yielding water. Similar reduced molecules can be imported from the cytosol via the malate-aspartate system or feed into the ETC via glycerol phosphate shuttles. When oxygen is limited, glycolytic products are metabolized by anaerobic respiration, a process that is independent of the mitochondria. However, the production of ATP from glucose has an approximately 13-fold higher yield during aerobic respiration, as compared to anaerobic respiration.

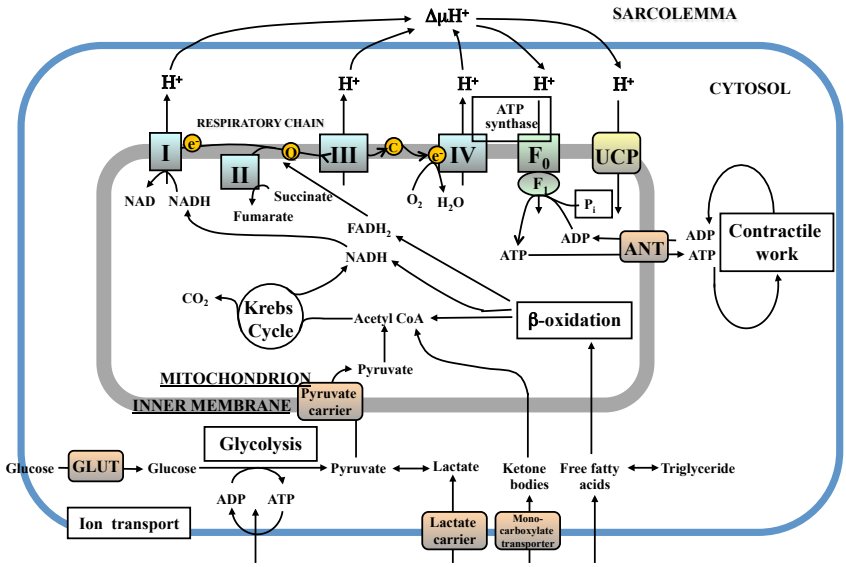

Figure I4. The mitochondrial electron transport chain (UCP- Uncoupling protein; GLUT- Glucose transporter; ATP- Adenosine triphosphate; ADP- Adenosine diphosphate; ANT- ADP/ATP translocator; FAD- Flavin adenine dinucleotide; NAD- Nicotinamide adenine dinucleotide).

The process of respiration is highly efficient; however, under certain conditions, a proton leak uncoupled from ATP synthesis (uncoupling) may reduce the generation of reactive oxygen species (ROS), such as superoxide. Such ROS are natural by-products of cellular metabolism, occurring in up to 2% of electrons passing through the ETC and have important roles in redox signaling (Lee and Griendling, 2008). However, when ROS levels are left unchecked, they can result in severe impairment of cellular function and ultimately lead to cell death. Uncoupling proteins (UCPs) are mitochondrial anion carriers found in the inner mitochondrial membrane and are one of the cellular mechanisms responsible for the regulation of cellular ROS levels (Rousset et al., 2004, Mailloux and Harper, 2011). UCPs are capable of uncoupling generation of ATP from the action of the ETC, by altering the potential difference across the mitochondrial membrane, resulting in the production of heat only from the consumption of oxygen. The mechanism of mitochondrial membrane potential alteration has been demonstrated to vary, but can occur via proton translocation or via cycling of protonated fatty acids across the mitochondrial membrane (Garlid et al., 1998, Jaburek et al., 1999). UCP 2 is a ubiquitous molecule, whereas UCP 3 is mainly expressed in skeletal muscle; however, both are activated by the presence of ROS, which modulate the

UCP molecule via covalent modification by glutathione, to provide a negative feedback loop for mitochondrial ROS production (Mailloux and Harper, 2011, Rousset et al., 2004, Negre-Salvayre et al., 1997).

Further mitochondrial functions include crucial roles in the reception and transmission of cellular signals; the regulation of membrane potential, cell differentiation, cycling, growth and apoptosis; haem and steroid synthesis, and ammonia detoxification within hepatic cells; and the regulation of reactive oxygen species' synthesis and tissue oxygen gradients. Mitochondria are also important regulators of local intracellular calcium ($[Ca^{2+}]_i$) and there is significant calcium interchange between mitochondria and ER (Szabadkai and Rizzuto, 2004), and the ability of mitochondria to rapidly accumulate and release $[Ca^{2+}]_i$ makes them effective calcium buffers for various cellular microdomains. Calcium is transported into the mitochondrial matrix by a calcium uniporter on the inner mitochondrial membrane (which is driven by the electrochemical gradient of the mitochondrial membrane potential), where it causes a transient depolarization in the mitochondrial membrane potential and directly or indirectly regulates both mitochondrial and cytoplasmic metabolic processes (Miller, 1998). Within the mitochondrion, calcium is crucial for the functioning of citric acid cycle dehydrogenase enzymes and has a role in mitochondrial respiration, dynamics and levels of oxidative stress (Saotome et al., 2008, Pandya et al., 2013). Calcium release from the mitochondria is triggered via the action of a sodium-calcium exchanger and a calcium-induced-calcium-release mechanism and subsequent alterations in intracellular calcium levels remain necessary for effecting various second messenger systems (Hajnoczky et al., 2002). Larger $[Ca^{2+}]_i$ surges, however, can result in significant alterations in mitochondrial membrane potential, potentially triggering apoptotic mechanisms, via collapse of the mitochondrial membrane potential, when a certain calcium threshold is surpassed (Rizzuto et al., 2009). Thus, fine regulation of calcium signaling is required and dysregulated mitochondrial calcium regulation has been implicated in multiple disease states (Decuypere et al., 2011).

I3. b. RAS Components Within Mitochondria

Mizuno *et al* (1988) performed bilateral nephrectomy on male Sprague-Dawley rats, a

technique which has been shown to completely eliminate circulating renin, whilst increasing renin and aldosterone synthesis (Doi et al., 1984), although the rise in aldosterone can be inhibited by administration of Losartan (Doi et al., 1984, Peters et al., 1999). The authors performed subcellular fractionation and percoll density gradient separation of the adrenal glands in combination with radioimmunoassay for protein and enzyme content. Immunohistochemical analysis revealed the presence of dense granules within zona glomerulosa cells, which increased in number following bilateral nephrectomy, and which stained for the presence of renin (see Figure I5). Similarly, following percoll density gradient centrifugation of adrenal capsules and immunogold staining, renin was found mainly in dense granules of mitochondrial fractions, where radioimmunoassay for renin activity was 15 times greater in nephrectomised rats than intact rats. The authors argued that these results provide evidence that cells of the adrenal gland synthesize renin and store it within specific secretory granules that can be located within mitochondria. However, their study methodology excluded organelles of high density (5600 g), which may have contained mitochondria and dense bodies; and no direct immunocytochemistry was performed.

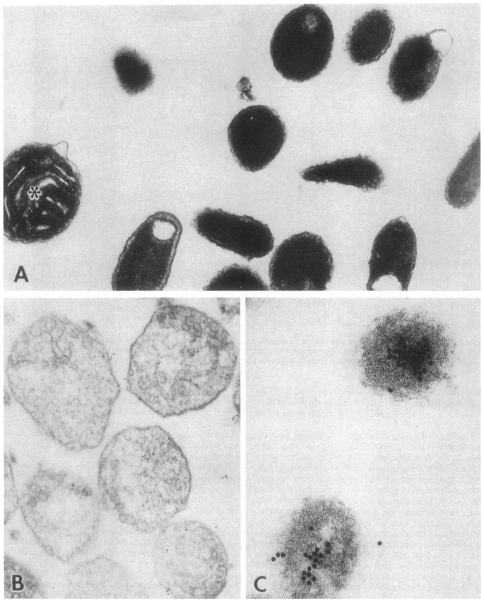

Figure I5. Electron micrographs of sections from Percoll density gradient fractions. (A) Electron-dense granules and a condensed mitochondrion (*) from high density fraction of nephrectomized rat adrenal fixed in glutaraldehyde osmium (x 72,000 magnification). (B) Mitochondria from low density fraction stained with anti-renin demonstrated no specific localization (x 49,000 magnification). (C) Granules in high density fraction stained with anti-renin demonstrate numerous particles overlying the granules (x 135,000 magnification) (Mizuno et al., 1988).

Peters *et al* (Peters et al., 1996) attempted to circumvent these drawbacks during their investigation of Sprague-Dawley rats undergoing bilateral nephrectomy and haemodialysis, with subsequent surgical excision and chemical post-fixing of adrenal glands. They exposed ultra-thin sections to antibodies against renin (recognising renin and prorenin), ACE and angiotensinogen, as well as performing differential centrifugation and separation of cellular organelles and determining prorenin, renin and angiotensinogen concentrations by radioimmunoassay. Following bilateral nephrectomy, plasma renin concentration decreased rapidly within ten hours, followed by a further decrease to undetectable levels within the next 20 hours. In contrast, plasma active renin in haemodialysed control rats increased. The levels of plasma prorenin increased in both control and nephrectomized rats; however, the addition of a specific rat renin inhibitor (CH-732) inhibited ang I generation in plasma of dialyzed control rats only (by 65%) and not in nephrectomized rats; the authors concluding that true active renin and prorenin were eliminated from the circulation and that any renin detectable in adrenal glands can therefore be attributed to local synthesis only as the animals were without circulating renin for at least 20 hours. Plasma angiotensinogen was strongly elevated by nephrectomy and remained elevated for the duration of the study period.

Utilising anti-renin antibodies (anti-mouse & anti-rat) and immuno-gold labeling, renin immunoreactivity was found over membrane-bound structures of moderate electron density within zona glomerulosa cells, the authors concluding that these most likely resembled immature renin vesicles of juxtaglomerular cells within the kidney and were therefore likely to represent storage or transport; and within mitochondria where gold staining was restricted to high density, rounded inclusion bodies (see Figure I6). These structures were further characterised and imaged by epon-embedding to improve morphology preservation (see Figure I7). In control rats, ACE was located within mitochondria particularly concentrated over intramitochondrial dense bodes; whereas angiotensinogen and cathepsin D (as a lysosomal marker) were only found within cytoplasmic vesicles. Bilateral nephrectomy led to an increase in renin particles within cytoplasmic vesicles and mitochondria, and the immunogold staining technique was believed to be specific as signals within both mitochondria and cytoplasmic vesicles were absent following preadsorption of anti-renin antiserum with purified renin.

Intracellular organelles of zona glomerulosa of both control and nephrectomised rats were separated by differential centrifugation, and their renin content was determined. Peak mitochondrial fractions were at 400-1000 g (as indicated by malate dehydrogenase concentration); whereas, peak lysosomal fractions were 3,000-15,000 g (as indicated by acid phosphatase concentration). The intracellular concentration of active renin was increased 10 x in anephric rats and two peaks of active renin were observed, one overlapping with the lysosomal fraction (the authors concluding that this probably represented cytoplasmic vesicles) and the other with a 600 g mitochondrial fraction (see Figure I8). Similar results were observed in control rats although renin concentrations were much lower and the mitochondrial peak, in particular, was barely detectable. Aliquots of the subcellular fractions were subsequently utilized for immunocytochemistry and renin immunoreactivity was again confirmed within mitochondrial fractions. Further investigation demonstrated ACE within mitochondria and mitochondrial dense bodies and angiotensinogen within cytoplasmatic vesicles of rat adrenal cortex of untreated Sprague-Dawley rats demonstrate. The authors thus concluded that the study provides evidence for the existence of an intracellular RAS in the adrenal cortex and further, that mitochondria here may be independently capable of synthesising RAS components, specifically renin (Peters et al., 1996). Although the study did not pursue the investigation of other RAS components, elimination of renin from the rat circulation prior to adrenal gland analysis provides good support for intra-mitochondrial RAS synthesis in adrenal cortex.

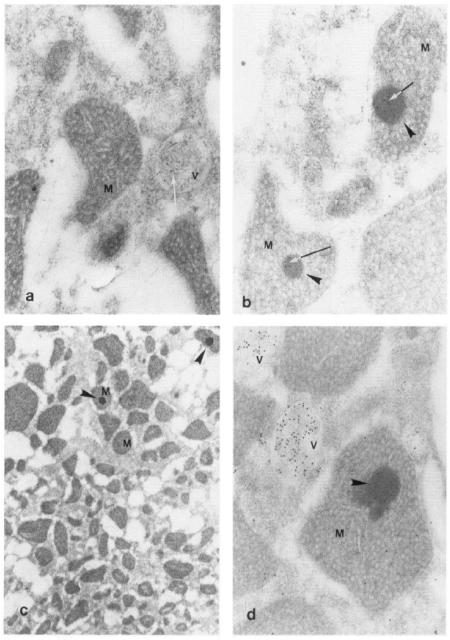

Figure I6. Electron microscopy and immunocytochemical localization within rat adrenal zona glomerulosa of Sprague-Dawley rats demonstrate cytoplasmic vesicles with immunogold signals (a; white arrow; magnification x 23,000); mitochondrial dense bodies containing renin (b; arrows; magnification x 23,000); intramitochondrial dense bodies (c; arrowheads; magnification x 4700); cathepsin D within cytoplasmic vesicles (d; arrowhead; magnification x 23,000) (Peters et al., 1996).

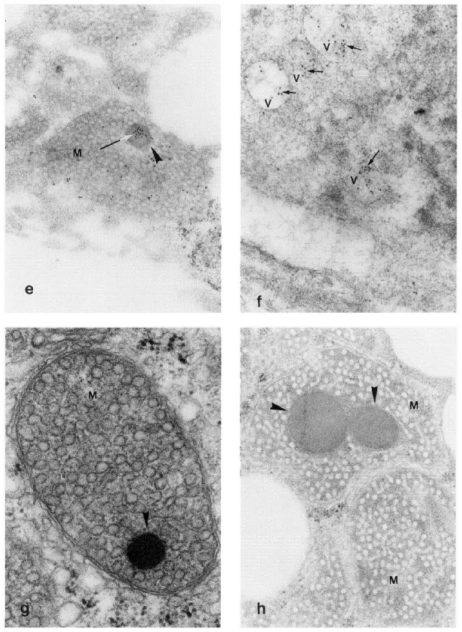

Figure I7. Electron microscopy and immunocytochemical localization within rat adrenal cortex of Sprague-Dawley rats demonstrate ACE within mitochondria and

mitochondrial dense bodies (e; arrowhead; magnification x 23,000); angiotensinogen within cytoplasmatic vesicles (f; arrows; magnification x 23,000); Epon-embedded adrenal section demonstrating dense body within mitochondria (g; arrowhead; magnification x 30,000); Tannin-prestained Epon-embedded adrenal section showing two dense bodies within the same mitochondrion (h; arrowheads; magnification x 23,000) (Peters et al., 1996).

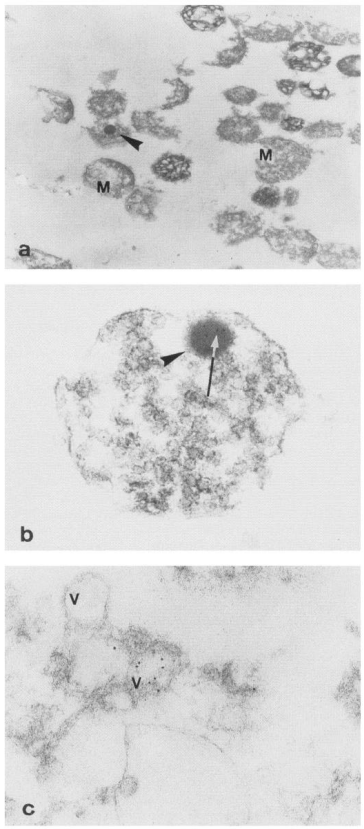

Figure I8. Immunocytochemistry of zona glomerulosa subcellular fractions (Peters et al., 1996).

a) Absence of vesicles and extramitochondrial dense bodies in 600 g mitochondrial fraction, thus demonstrating no contamination of the fraction. Multiple mitochondria are present, one containing an intramitochondrial dense body (Arrowhead; magnification x 10, 000). **b)** Immunocytochemistry of 660 g mitochondrial fraction demonstrating renin immunoreactivity (arrow; magnification x 23, 000). **c)** 9000 g fraction containing vesicles, one of which has renin immunoreactivity (magnification not supplied).

The rat cerebellum contains all components of the renin-angiotensin system (Lippoldt et al., 1994) and ang II has been shown to act as both a neurotransmitter and neurohormone (Fuxe et al., 1976, Lind et al., 1985). Further, titrated ang II, when administered to rodents, traffics to AT_1Rs on the outer mitochondrial membrane of the rat sensory vagal complex (Robertson and Khairallah, 1971b, Re, 2003, Huang et al., 2003). Erdmann *et al* (1996) therefore extended these previous findings to investigate the presence of RAS components in rat cerebellar neurons. The authors excised and fixed the brains of 15 male Sprague-Dawley rats followed by processing for conventional cryostat or cryoultramicrotomy and staining for ang II (monoclonal and polyclonal antibodies). Different fixatives led to different appearances of ang II: formaldehyde and picric acid led to a patchy yellow-brownish staining in different layers of the cerebellar cortex, especially the granular layer; whereas, the addition of glutaraldehyde to the fixative revealed a nuclear localization of ang II mainly in Purkinje cells (see Figure I9). However, when semi-thin cryosections were utilized rather than conventional cryostat techniques, improved structural preservation allowed clear identification of ang II immunoreactivity in the nuclei of Purkinje, granule, basket, and stellate cells. Ang II immunoreactivity was also sporadically identified in the cytoplasm of different cell types, in vesicle-like structures of the cytoplasm and in mitochondria. Preadsorption of the primary antibody abolished the ang II immunolabeling in nuclei and cytoplasm of all cell types but within mitochondria approximately one third of the labeling remained unchanged even after preadsorption. The authors concluded that when taken in the context of previous studies that have demonstrated other RAS components, such as renin and ACE (Fuxe et al., 1980, Chai et al., 1987), the findings provide good evidence for the presence of intraneuronal generation and processing of ang II, particularly in Purkinje cells. However, immunohistochemical identification of small molecular weight antigens relies upon the fixative inducing a coupling reaction with tissue proteins, leading to immobilization of the substance studied (Soinila et al., 1992); but within the brain, the presence of the blood-brain barrier means that all areas are not directly accessible to the bloodstream and that fixation may therefore be incomplete. Significant redistribution of the antigen under investigation may therefore occur during fixation and subsequent processing, meaning that results are not necessarily representative of true locations (Slot et al., 1989). Further, previous studies also demonstrate that angiotensinogen immunoreactivity within the rat brain is highly dependent upon the tissue treatment and sectioning techniques employed and the methods employed within the study may have therefore similarly affected study

results (Campbell et al., 1991). In keeping with this is the fact that mitochondrial labeling in this study could not be abolished after preadsorption of the primary antibody with the peptide and therefore may contain a significant amount of nonspecific binding.

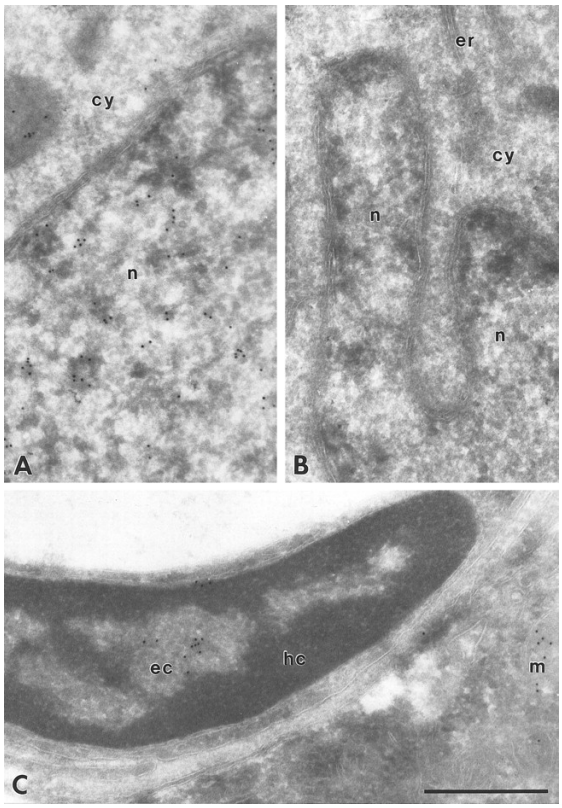

Figure I9. Electron Microscopy and immunocytochemical localization (monoclonal antibody) of ang II in: nucleus of purkinje cell and vesicle-like structures within cytoplasm (A); control image with no staining (B); ang II labelling of endothelial cell nucleus, preferentially of euchromatin, and in mitochondria abutting neuropil (C) (magnification x 70,000; N-nuclei; c- Cytoplasm; m- Mitochondria; ec- Euchromatin) (Erdmann et al., 1996).

The mitochondrial genome contains no genes for RAS components and the discovery of renin and other RAS components within mitochondria therefore raised the question of how the proteins enter the mitochondria. Clausmeyer et al (1999 and 2000) hypothesized that mitochondrial forms of RAS components must be derived from the nuclear genome, synthesized within the cytosol and imported following post-

translational modifications. The authors further suggested that the differential targeting of renin to mitochondria, rather than to a secretory pathway, might occur via an additional downstream initiation of translation, resulting in the lack of an ER signal sequence and escape from the secretory pathway. They therefore constructed amino-terminal deletion mutants of prorenin, active renin and several intermediate forms, by PCR, and analysed them in conjunction with isolated mitochondria of the rat adrenal gland, as well as characterizing several transcripts of the renin gene. Of the six renin variants, only two were transported with considerable efficiency into mitochondria (D26 and D36), both of which lacked the complete ER-targeting sequence of preprorenin and they concluded that a form of renin lacking 36 amino acids, namely the complete ER signal sequence, and 10 amino acids of the profragment, meets the requirements for mitochondrial import. There was no observed processing of the imported renin variants, indicating that the common proteolytic cleavage of an amino-terminal targeting sequence upon import into the mitochondrial matrix, did not occur and that these proteins may have contained an internal targeting signal instead, which may lie between amino acids 36 and 50; although this region differed from mitochondrial targeting sequences in that it contained some negatively charged amino acids, alongside three arginine residues. However, a number of mitochondrial proteins contain an internal targeting sequence that is not cleaved (e.g. cytochrome c (Folsch et al., 1996, Nicholson et al., 1988, Lill et al., 1992)) and are usually located within the inner membrane or inter-membrane space. However, renin has been located within dense bodies, which are most likely to be located within the mitochondrial matrix, requiring transport across the inner membrane and further investigations are required to demonstrate activity of such renin proteins in vivo. Analysis of renin mRNA also identified two transcripts with different 5'- regions: besides the full-length transcript, an additional mRNA lacking exon 1, but containing an 80-nucleotide region having its origin in intron 1, was discovered. The authors concluded that this represented an alternative spliced transcript, in which exon 1 appears to be replaced by another, previously undescribed exon (exon 1A). However, besides a possible role in the regulation of mitochondrial aldosterone production, further functions of this novel renin remain unknown.

The same group went on to extend their observations of this novel non-secretory, cytosolic renin transcript (exon(1A-9) renin), which is translated into a truncated prorenin commencing at the first-in-frame ATG (translation start site) in exon 2, resulting in a protein that lacks the prefragment of secretory renin as well as the first 10 amino acids of the conventional protein. In the rat most tissues have been found to express both transcripts, the kidney exon(1-9) only and the heart expresses the exon(1A-9) transcript exclusively, where its levels increase after myocardial infarction (Clausmeyer et al., 2000; Wanka et al., 2009). The authors therefore over-expressed both conventional 'secretory' renin (exon(1-9) renin) and novel 'cytosolic' renin (exon(2-9) renin) in H9c2 cells (a rat embryonal cardiac muscle-derived cell line) and subsequently performed confocal microscopy after double-staining for renin and mitochondrial markers, as well as performing subcellular fractionation and assessing ang I concentration by radioimmunoassay as a measure of renin's ability to generate ang I. In H9c2 cells expressing exon(1A-9) renin, renin immunoreactivity was found exclusively within mitochondria (see Figure I10). Following subcellular fractionation, inactive renin was enriched in a 200 g mitochondrial fraction (as confirmed by the presence of the mitochondrial marker malate dehydrogenase) in non-transfected H9c2 cells. Over-expression of exon(2-9) renin protein led to increased inactive and active renin concentrations in the 200 g mitochondrial fraction; whereas, in a second light mitochondrial fraction (3000 g), inactive, but not active, renin was also increased. Following over-expression of exon(1-9) renin, inactive renin was enriched in a 15,000 g light vesicular fraction only. Inactive and active renin concentrations were also increased in the medium of exon(1-9) cells, but not in exon(2-9) transfected cells supporting the hypothesis that exon(1-9) renin is secretory, but exon(2-9) is cytosolic. Cellular proliferation rate was reduced by secretory and cytosolic renin, necrosis was increased by secretory renin but decreased by cytosolic renin, and mitochondrial apoptosis, as indicated by phosphatidylserin translocation to the outer membrane, was unaffected by secretory renin but increased by cytosolic renin.

The authors concluded that renin is sorted to different intracellular compartments within H9c2 cells dependent upon the presence of exon 1 or 1A and the truncated exon(2-9) renin protein encoded by the exon(1A-9) transcript lacks 10 out of 43 amino acids of the profragment of secretory prorenin. Further, the authors report that, since cytosolic renin is up-regulated following myocardial infarction, it may play a protective role in ischemia-related processes by reducing the rate of necrosis and increasing apoptosis, thereby

preventing unfavourable fibrosis and cardiac remodeling.

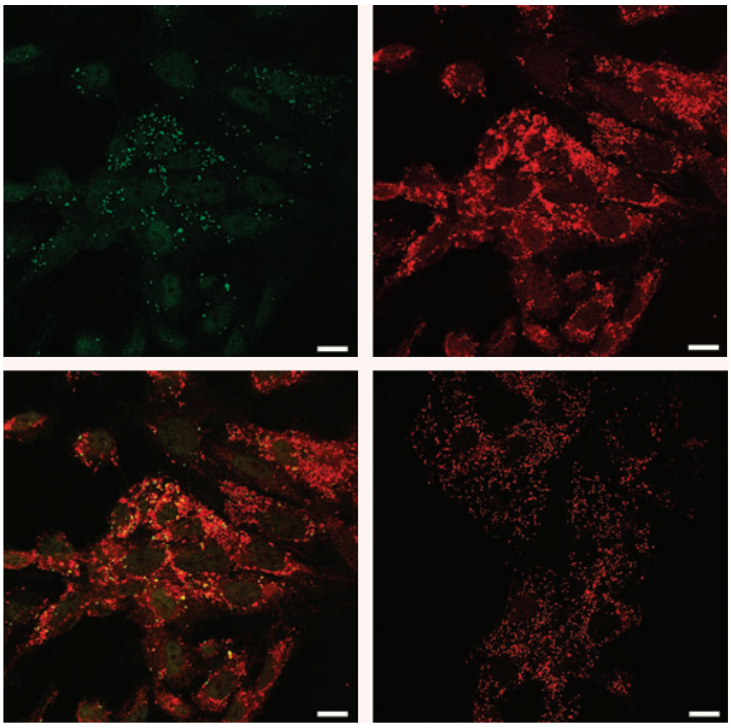

Figure I10. Immunohistochemical localization of renin in H9c2 cells. Anti-renin antibody (upper left panel, green); Mitotracker Red for mitochondrial staining (upper right panel, red). Merged confocal images demonstrated that renin co-localizes to mitochondria (lower left panel; colocalization: yellow). Merged confocal image of non-specific rabbit antiserum and Mitotracker Red as negative control (lower right panel) (Wanka et al., 2009).

Abadir *et al* (2011) investigated the role of the RAS in intra-mitochondrial nitric oxide (NO) production by performing immunoelectron microscopy for various RAS components in sections from human skeletal muscle, cardiac myocytes, renal tubular cells, neuronal cells, vascular endothelial cells and hepatocytes, as well as transfecting human fibroblasts with an AT_2R construct, performing subcellular fractionation, and measuring mitochondrial NO production and respiration. Electron microscopy demonstrated that AT_1R immunoreactivity was present on monocyte cell membranes, but was not regularly present in mitochondria from young monocytes or tubular renal cells; whereas AT_2R immunoreactivity was present in human monocyte cell membranes and mouse tissues (see Figures I11-14). Mouse heart

homogenates were subsequently fractionated by differential centrifugation and density gradient separation to demonstrate that AT_2R immunoreactivity increased with progressive mitochondrial purification as indicated by cytochrome c oxidase activity. Double-labeling also demonstrated co-localisation of AT_2R and Ang within mouse hepatocytes, kidney tubular cells, brain neurons, and heart myocytes. The authors report that further inspection of the immunoelectron images suggested that mitochondrial AT_2R is present on the inner mitochondrial membrane so proceeded with isolating inner mitochondrial membrane-enriched preparations from mouse liver to demonstrate that AT_2R immunoreactivity co-purified with ATP synthase, a marker of the inner mitochondrial membrane. Further, following transfection of human fibroblasts with a GFP-AT_2R construct and subsequent counterstaining with Mitotracker Red to indicate mitochondria, significant overlap was found with a high spatial correlation (R^2=0.72). NO production was also dose-dependently activated by the AT_2R agonist CGP421140 in isolated mitochondria, and this effect was prevented by AT_2R blocker PD-123319 and the fact that mitochondrial respiration and membrane potential were unaffected means that observed effects were unlikely to be attributable to non-specific toxic effects of any of the added substrates upon mitochondrial energetics. However, the use of a single antibody for the assessment of AT_2R renders the study potentially open to methodological limitations and the mitochondrial immunogold and immunofluorescence labeling results reported by Abadir *et al* (2011) should thus be interpreted with significant caution. Moreover, the study applied only CGP 42112, an AT_2R agonist, which has been reported to antagonise the AT_1R as well (Brechler et al., 1993), and functional impacts of natural AT receptor ligands were not demonstrated. Further, different experiments were performed in different cell types and tissues and therefore the consistency of such results in one cell type cannot be proven.

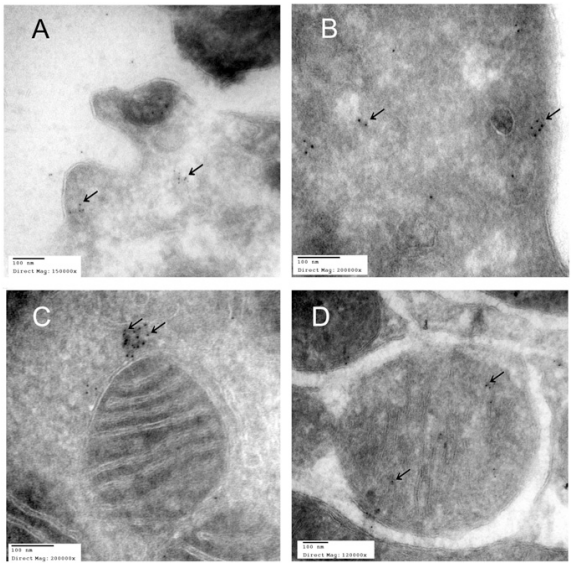

Figure I11. Immunoelectron microscopic localization of AT_1Rs in sections of human monocyte (A and B) and mouse kidney tubular cells (C and D) using gold bead labeling (arrows). (A) AT_1Rs are present on human monocyte cell membrane; (B) AT_1Rs are present in the cytoplasm; (C) AT_1R labeling is present in close proximity to mitochondria; (D) AT_1Rs are rarely present within mitochondria (Abadir *et al*., 2011).

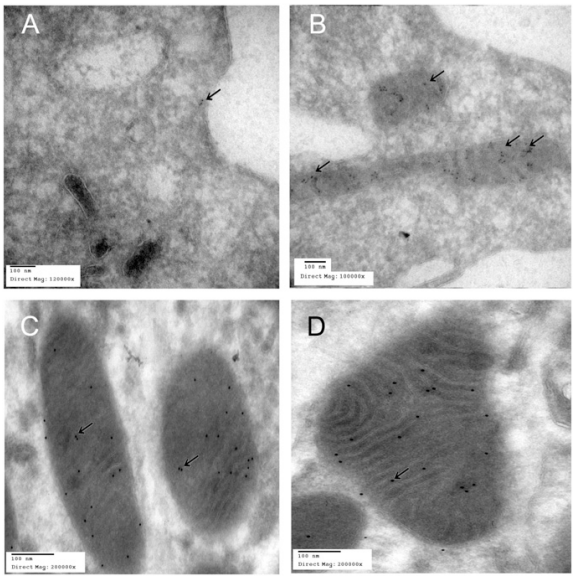

Figure I12. Immunoelectron microscopic localization of AT_2Rs in sections of human monocyte (A and B) and mouse renal tubular cells (C and D) using gold bead labeling (arrows).

(A) demonstrates AT_2Rs on human monocyte cell membrane; (B–D) demonstrate heavy AT_2R staining within mitochondria (Abadir *et al*., 2011).

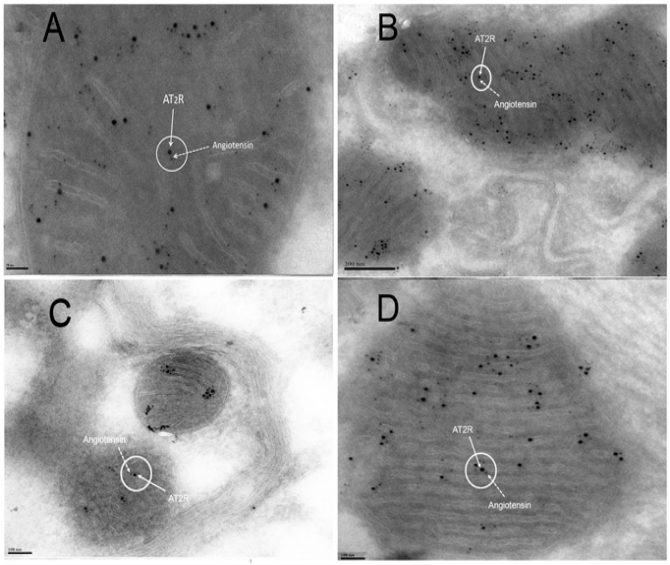

Figure I13. Immunoelectron microscopic localization of AT_2R binding to Angiotensin within mitochondria utilising a gold-labeled anti-AT_2R antibody (12 nm gold) and a gold-labeled anti-Ang antibody (6 nm gold). Shown is colocalization of AT_2Rs with Ang in sections of (A) mouse hepatocytes; (B) kidney tubular cells; (C) neurons; (D) cardiac myocytes (Abadir *et al*., 2011).

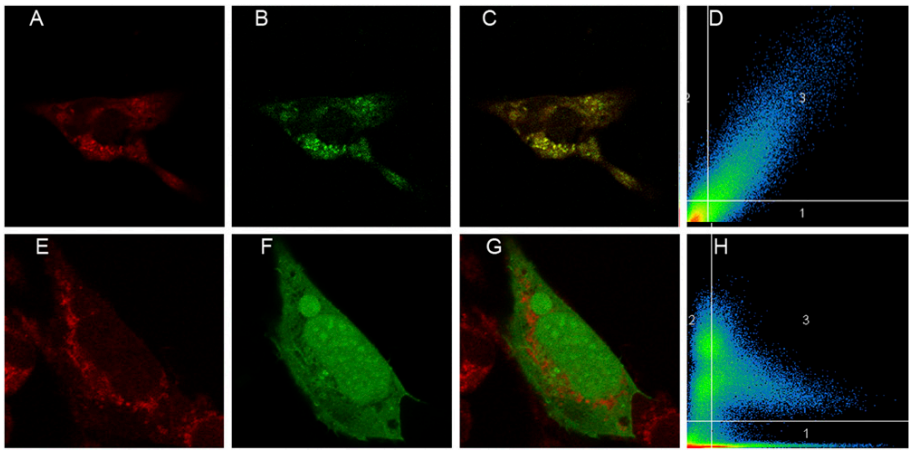

Figure I14. Transfected AT_2Rs colocalize with mitochondria in human fibroblasts.
(B) Human fibroblast cells were transfected with GFP-AT_2R construct or positive control (F) and counterstained with MitoTracker Red (A & E). The merged images show yellow fluorescence (C & G). Fluoro-graphic analysis (D & H) reveals a high correlation coefficient ($R^2 = 0.72$) (Abadir *et al*., 2011).

I3. c. RAS Effects Upon Mitochondria

I3. c. i. Mitochondrial Morphology & Number

Long-term administration of ang II to rats leads to significant decreases in mitochondrial number (de Cavanagh et al., 2007, Basso et al., 2007) and reduced expression of mitochondrial metabolism genes such as those involved in the Krebs cycle (Larkin et al., 2004). It also mediates age-related structural changes (e.g. loss of cristae, mitochondrial swelling and decreased overall number of mitochondria) within mitochondria (de Cavanagh et al., 2007, Basso et al., 2007). RAS-inhibition (via AT_1R blockers or ACE-I), however, is capable of reversing these effects (Chen et al., 2004, de Cavanagh et al., 2003, De Cavanagh et al., 2005, de Cavanagh et al., 2006, de Cavanagh et al., 2008a), preserving mitochondrial number and morphology, and reversing age-related structural changes (Basso et al., 2007). This also upregulates expression of energy production genes (mitochondrial ETC and krebs cycle genes) in diabetic mice (Chen et al., 2004). Further genes downregulated by ang II action at the AT_2R include those involved in mitochondrial biogenesis, leading to reduced mitochondrial content and mitochondrial membrane potential in C2C12 (mouse myoblast cell-line) cells and reduced mitochondrial content in mouse skeletal muscle (Mitsuishi et al., 2009).

Ang II may also induce integrin signaling, resulting in the release of transforming growth factor-1 (TGF-1), modification of extracellular matrix (ECM) composition and structure, and cytoskeleton reorganisation (de Cavanagh et al., 2009). Mitochondria rely upon cytoskeletal dynamics for physiological motility, morphology and localization, and may therefore have secondary effects upon mitochondrial function, potentially contributing to pathological conditions, such as cardiovascular and renal fibrosis (de Cavanagh et al., 2009).

I3. c. ii. Age-Related Changes & Life-Span

Ang II may act as a mediator of the aging process by augmenting mitochondrial oxidant damage. The administration of AT_1R blockers or of ACE-I reduces oxidant production,

preserves mitochondrial number and morphology, and prevents the reduction in mitochondrial DNA content associated with aging. AT_1R deficient mice demonstrate a 25% longer life-span than mice with AT_1 receptors (mean life span 31.20±2.31 vs. 24.81±3.10 months) (Cassis et al., 2010). The aging process is frequently associated with a reduction in mitochondrial number and changes in mitochondrial structure, such as swelling, shortening of the cristae, and matrix vacuolization, and therefore lowering RAS levels may prolong life by preventing such changes. Such AT_1R knockout mice also have increased numbers of mitochondria; upregulation of pro-survival genes sirtuin 3 (NAD-dependant deacetylase important in ageing, stress resistance and regulation of metabolism) and Nampt (nicotinamide phosphoribosyltransferase, a glycosyltransferase involved in nicotinamide metabolism); decreased end-organ damage, cardiovascular injury and atherosclerosis; and decreased ROS and peroxynitrite despite normal body-weight, physical activity and food intake (Benigni et al., 2009, Cassis et al., 2010).

I3. c. iii. RAS and Reactive Oxygen Species

Ang II increases mitochondrial ROS production and may contribute to oxidative stress damage by uncoupling endothelial nitric oxide synthase, switching generation of nitric oxide (NO) to superoxide production (via NF-kappaβ) (Dikalova et al., Kimura et al., 2005). Ang II also binds to the AT_1R, leading to generation of ROS within the cytosol, through PKC-dependent (protein kinase c) activation of NAD(P)H Oxidase (Doughan and Dikalov, 2005). Subsequent interaction between NO and superoxide generates peroxynitrite, a cytotoxic anion that inhibits mitochondrial electron transport, as well as destroying DNA and cellular proteins. Such highly reactive oxygen species can create a positive feedback loop by further activation of cellular NADPH oxidase (see Figure I15) and can also directly inactivate mitochondrial complexes, possibly via tyrosine nitration, and result in uncoupling of oxidative phosphorylation (Vatassery et al., 2004). Ang II similarly increases mitochondrial ROS and superoxide production in neurons, which mediates ang II-induced activation of calmodulin kinase II and inhibition of neuronal potassium current (Yin et al., 2010).

Thus, ang II stimulates both cytosolic and mitochondrial reactive oxygen species (ROS) generation, as well as altering O_2 consumption and contributing to cachexia (Brink et al.,

1996, Cassis et al., 2002). Selective blockade of NADPH oxidase (by apocynin) or PKC (by chelerythrine) dramatically attenuates mitochondrial ROS generation in response to ang II within isolated mitochondria or intact bovine aortic endothelial cells (Doughan et al., 2008). Meanwhile, SiRNA (small interfering RNA) depletion of p22phox, an essential component of NADPH Oxidase, leads to further significant decreases in mitochondrial ROS production in mitochondria isolated from ang II-treated cells (Doughan et al., 2008). Ang II administration also results in reductions in mitochondrial glutathione, the major endogenous antioxidant (which can be prevented with RAS antagonists) (Vatassery et al., 2004, Pompella et al., 2003, Doughan et al., 2008), potentially sensitizing mitochondria to further ROS damage, as well as diminishing mitochondrial respiratory control ratio (Doughan et al., 2008).

Thus, under normal physiological conditions, the capacity of ang II to promote oxidative stress is tightly regulated. By contrast, in conditions associated with RAS overactivation, such as hypertension, diabetes and ageing, dysregulation of ang II-dependent ROS generation may become a significant contributor to cell oxidation and tissue damage, as well as reduction of bioenergetic synthesis and derangement of mitochondrial ROS-mediated signals. In keeping with this, ang II administration to Ren2 rats (which over-express renin, and exhibit elevated endogenous ang II levels) causes ultrastructural abnormalities, decreased mitochondrial content, decreased palmitate oxidation, decreased enzymatic activity and decreased levels of cytochrome c, cytochrome c oxidase subunit I, and mitochondrial transcription factor A (Wei et al., 2009). These effects are ameliorated by ARB administration, substantially attenuating mitochondrial lipid peroxidation in hepatic tissue and preventing hepatic steatosis (Wei et al., 2009).

I3. c. iv. RAS and Mitochondrial Function (see Figure I15)

Isolated mitochondria from rats treated with the ACE inhibitor enalapril exhibit significantly lower energy transfer through complexes I to III (measured as the ratio of NADH/cytochrome *c* oxidoreductase activity), as well as lower cytochrome oxidase activity and higher UCP-2 content (Piotrkowski et al., 2007), with the authors suggesting that these

data demonstrate a direct effect of ACE inhibition upon mitochondria; however, no mechanism for such action, and no further discussion of why a reduction in energy transfer at the ETC would be beneficial, was postulated.

In treated heart failure patients, cardiac oxidative capacity was found to be 25 % lower than in patients without heart failure; however, ACE-I was capable of partially protecting cardiac mitochondrial function (oxygen consumption measured in fresh saponin-skinned fibers from rat left ventricle) and transcription cascade (downregulation of expression of multiple mitochondrial genes encoded by both nuclear and mitochondrial genomes, including mitochondrial transcription factor A and COX) in a rat model of myocardial infarction (Garnier et al., 2009). Rats treated with a low (no effects upon blood pressure) dose of enalapril demonstrated lower MMP-2 (Matrix Metalloproteinase 2) activity, but higher levels of cytochrome c oxidoreductase activity, eNOS protein and activity and mtNOS activity (Piotrkowski et al., 2009).

Ang II also depolarises mitochondrial membrane potential in islated mitochondria from bovine aortic endothelial cells (Dikalova et al., 2010); whereas, Losartan treatment results in an increased rate of ATP production and in elevated concentrations of coenzyme Q9 and Q10 (Sumbalova et al., 2010). Recent novel findings also suggest that ang II stimulates opening of mitochondrial K^+ATP channels, depolarizes mitochondrial membrane potential and increases mitochondrial ROS production, resulting in activation of redox-sensitive MAPK (mitogen-activated protein kinase) (Zhang et al., 2007).

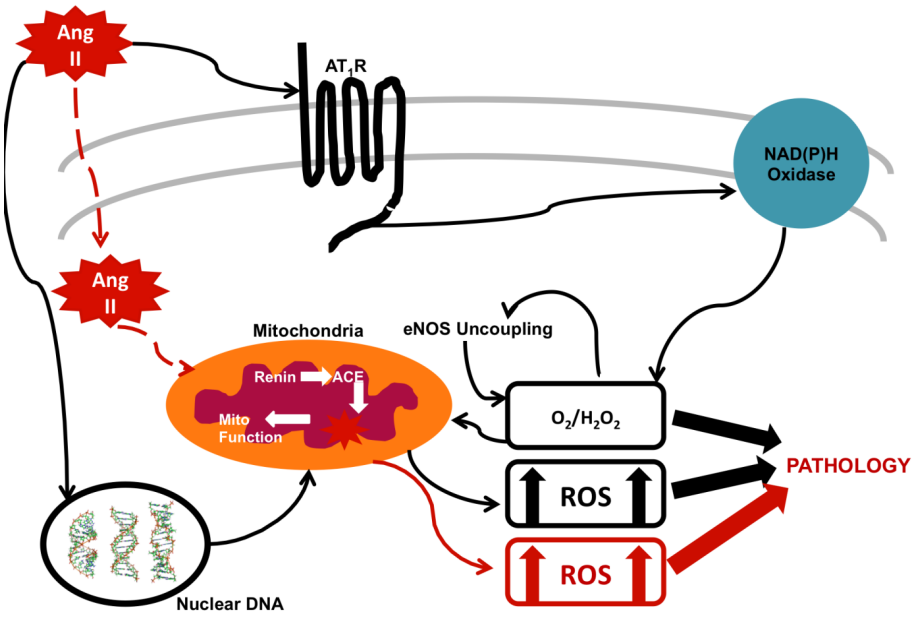

Figure I15. Ang II induces endothelial Nitric Oxide Synthase uncoupling, switching from Nitric Oxide to Superoxide production, as well as potentially having direct action upon mitochondrial reactive oxygen species production; a pathway that occurs even under normal physiological conditions (pathway in black), but when the RAS pathway is increasingly activated to above physiological levels (pathway in red), it may lead to various pathological states.

I4. Summary & Rationale

I4a. Summary of Published Literature

Ang II has been detected in rat heart, brain and smooth muscle cell mitochondria, utilising 125-I labeled ang II (Robertson and Khairallah, 1971a, Sirett et al., 1977), and ang II immunoreactivity has also been observed in rat cerebellar cortex mitochondria via immunogold staining techniques (Erdmann et al., 1996). Further, in rat adrenal zona glomerulosa, renin, angiotensinogen and ACE have been detected within intramitochondrial dense bodies (Peters et al., 1996). Such data suggest the intramitochondrial generation of ang II, which might have direct effects on mitochondrial function. In support, ang II can stimulate production of mitochondrial reactive oxygen species in vascular smooth muscle cells and in rat aorta *in vivo* (Kimura et al., 2005), via NAD(P)H oxidase activation (Doughan and Dikalov, 2005). Recent observations also reveal that ang II administration leads to a decrease in mitochondrial membrane potential, possibly as a result of ROS generation (Kimura et al., 2005). RAS antagonists are capable of preventing or reversing many of these effects: ACE-inhibitors prevent age-related changes in mitochondrial number and form in murine renal cells, myocardiocytes and hepatocytes (Ferder et al., 1993, de Cavanagh et al., 2007, de Cavanagh et al., 2003). Furthermore, the expression of genes related to mitochondrial energy production is up-regulated in captopril-treated diabetic rats (Chen et al., 2004).

Therefore, studies to date appear to indirectly suggest that a mitochondrial RAS exists, and that it may contribute to physiological mitochondrial function and pathophysiological mitochondrial dysfunction. Ang II antagonism, meanwhile, is not only capable of preventing these effects, but also improves mitochondrial function. However, the precise mechanisms underlying such mitochondrial effects require further elucidation and might be mediated through diverse cellular signaling pathways, or alternatively via direct effects upon mitochondria, as suggested by the presence of RAS components in/on mitochondria.

I4b. A Mitochondrial RAS: Unanswered Questions

The mitochondrial genome does not contain DNA for any RAS components and therefore it must be inferred that RAS components located within mitochondria have been nuclear-encoded and imported from the cytoplasm (Neupert, 1997). Indeed, many mitochondrial proteins are nuclear-generated, containing an amino-terminal mitochondrial localization sequence that allows mitochondrial delivery via cytosolic chaperones, where receptor-mediated ATP-dependent uptake occurs (Roise and Schatz, 1988). However, no RAS components have been shown to be synthesized with such a targeting sequence, although the identification of a number of nuclear-encoded mitochondrial transmembrane proteins without such a targeting sequence (such as adenine nucleotide translocase) and some that contain an altered targeting sequence (e.g. CYP11B1 and P450SCC) does not completely exclude the mitochondrial import of RAS components (Pfanner et al., 1987, von Heijne et al., 1989).

Renin is the most investigated putative mitochondrial RAS component to date and traditionally was considered to be a secretory protein targeted to the secretory pathway via the ER and golgi apparatus, a pathway that does not allow segregation of renin or its precursors to the mitochondria. However, multiple renin transcripts are now known to exist, one of which has been shown to contain an amphiphilic structure that may be capable of acting as a mitochondrial import sequence for receptor-mediated mitochondrial uptake. Further, some renin transcripts are now known to contain alternative start sites that may contain an extended amino-terminal with high hydrophobicity that could be used for mitochondrial import (Dzau et al., 1988). Another possibility is the existence of an internal targeting signal, such as that found in cytochrome c, which is not cleaved (Folsch et al., 1996, Nicholson et al., 1988, Lill et al., 1992). However, renin has been identified in mitochondrial dense bodies (Peters et al., 1996), which are likely to be located within the mitochondrial matrix, requiring transport across both the mitochondrial inner and outer membrane; whereas, proteins with an internal targeting sequence are usually located on the inner membrane or in the inter-membrane space (Clausmeyer et al., 1999).

Similarly, Abadir *et al* (2011) reported the presence of the AT_2R on mitochondrial inner membranes; however, the mechanism required for the transport of a seven transmembrane domain G-coupled receptor across the outer mitochondrial membrane remains unknown.

AIMS & PLAN OF STUDY

A1. Aims

The identification of a functioning mitochondrial RAS (mRAS) may aid in understanding the mechanism of interaction between mitochondria and various disease states, and existing data suggest the association of individual RAS components with mitochondria, where they may influence mitochondrial function. However, such studies are few and contain inherent and multiple limitations at a basic level: they address a limited range of cells only, have often relied upon immunocytochemistry/immunohistochemistry alone to demonstrate RAS components, have rarely sought to identify the co-existence of multiple RAS components, and have failed to seek the presence of some entirely. Thus, the existence of a putative mitochondrial RAS remains in doubt and further exploration, utilizing alternate techniques, is required.

These issues were addressed by determining the presence of all major RAS components in the mitochondria of liver cells (from rat liver tissue and liver cell-line, rather than just cell-line alone; contain high density of mitochondria) utilizing a number of antibodies (rather than relying on a single antibody) and techniques (rather than immunocytochemistry/ immunohistochemistry alone).

A2. Hypotheses

The hypotheses tested were:

1. RAS components can be found within mitochondria derived from rat liver tissue and a liver cell-line, utilizing Western blotting, confocal microscopy and electron microscopy techniques.

2. Addition of RAS components to liver cells adversely affects a variety of hepatic mitochondrial functions (as assessed via measurement of oxygen consumption, mitochondrial membrane potential, NADH concentration and calcium concentration); whereas, RAS antagonism leads to improvements in hepatic mitochondrial function.

A3. Experimental Plan

A3.i. Investigation of the Presence of RAS Components within Hepatic Mitochondria

Initially, a plan was devised to investigate the presence of RAS components within rat liver tissue and a liver cell-line utilizing the following techniques and chronology.

A3.i. a. Western Blotting: Western blotting was performed upon subcellular fractions (produced via differential centrifugation), including mitochondrial fractions, isolated from both rat liver tissue and a liver cell-line. The purity of each subcellular fraction was assessed by staining for proteins known to be located within each specific sub-fraction. The presence of all RAS components was subsequently assessed in each sub-cellular fraction. Multiple antibodies (from multiple manufacturers) to each RAS component were sourced and utilized to assess the specificity and sensitivity of such antibodies and to avoid the problems associated with the use of single antibodies. The optimal antibodies for each RAS component were subsequently utilized for confocal and electron microscopy.

A3.i. b. Immunofluorescence & Confocal Microcopy: Immunofluorescence and confocal microscopy was performed upon whole cells from a liver cell-line and the presence of all RAS components was assessed. The intra-cellular and intra-mitochondrial location of RAS components was subsequently investigated and co-staining for known nuclear (DNA) and mitochondrial (ATP Synthase) constituents was performed to act as positive controls. Further negative control images were taken by performing staining with primary and secondary antibody alone.

A3.i. c. Immunogold Staining and Electron Microscopy: Immunogold staining and electron microscopy was performed upon rat liver tissue and a liver cell-line and the presence of all RAS components were assessed. Negative control images were taken by performing staining with primary and secondary antibody alone.

A3.ii. Investigation of the Effects of RAS Agonism/Antagonism upon Mitochondrial Function

Subsequently, I planned to investigate the result of adding RAS agonists and antagonists to live cells from a liver cell-line, utilizing the following techniques and chronology.

A3.ii. a. Functional Fluorescence Microscopy: The effect of adding angiotensin II, losartan and PD123319 upon cellular NADH and calcium concentration, and mitochondrial membrane potential, was assessed.

A3.ii. b. Oxygen Consumption: The effect of adding angiotensin II, losartan and PD123319 upon oxygen consumption was assessed.

METHODS

M1: Materials

M1. a. Dyes & Probes

M1. a. i. Tetramethylrhodamine, methyl ester (TMRM) (Invitrogen Molecular Probes, Carlsbad, CA, USA)

TMRM is a cationic, mitochondrial-selective probe that is utilised for labeling and measuring mitochondrial membrane potential within intact, living cells via confocal microscopy, flow cytometry or fluorometric measurements (Floryk and Houstek, 1999) . Due to its optical and chemical properties, alterations in mitochondrial membrane potential are observed as variations in fluorescence intensity but not in emission spectra (Loew et al., 1993), thus distinguishing between signals from probe located within mitochondria, and signals from probe situated within other cellular compartments. Such changes in fluorescence intensity are usually expressed relative to control measurements rather than as exact values, which can be difficult to accurately determine. It is added in low concentrations (see discussion of specific experiments for exact concentrations used) to avoid aggregation and fluorescence quenching and is subsequently excited at 500 nm. This approach allows sensitive measurements of changes in mitochondrial membrane potential and TMRM can be utilised in conjunction with confocal microscopy to perform analyses over time, or to assess the effects of adding or inhibiting certain molecules.

M1. a. ii. Acetoxymethyl ester Fluo-4 AM (Invitrogen Molecular Probes, Carlsbad, CA, USA)

Fluo-4 is a green-fluorescent probe that binds to calcium and is utilised for measurement of calcium concentration within living cells. It is commonly administered as the non-fluorescent Fluo-4 AM, which is subsequently cleaved within the cell to produce free, fluorescent Fluo-4, which has high cell permeability, undergoes 40-100 x increase in fluorescence intensity upon calcium binding and is excited at wavelengths of 488 nm (Gee et al., 2000). It can similarly be observed with confocal microscopy in analyses of calcium concentration alterations over time.

M1. a. iii. Hoescht 33258 (Sigma-Aldrich, St. Louis, MO, USA)

Hoescht 33258 is a water-soluble, cell-permeable, Bis-benzimide dye, which binds to the minor groove of double-stranded deoxyribonucleic acid (DNA), particularly those sequences rich in adenine and thymine (Portugal and Waring, 1988). It can be utilised to stain DNA in live or fixed cells (Latt et al., 1975, Latt and Stetten, 1976) and is excited by ultraviolet light at 350 nm, emitting a blue fluorescent light at an emission maximum of 461 nm.

M1. b. Reagents

M1. b. i. Rotenone (Sigma-Aldrich, St. Louis, MO, USA)

Rotenone is a water-insoluble, respiratory chain inhibitor that blocks respiration in the presence of ADP by inhibiting the transfer of electrons from iron-sulphur centres in complexes I and II of the electron transport chain to Coenzyme Q_{10} (ubiquinone) (Kuznetsov et al., 2008). Coenzyme Q_{10} is fat-soluble and acts as a mobile electron carrier in the mitochondrial membrane, delivering electrons to complex III and acting as a crucial cog in the electron transport chain machinery.

Coenzyme Q_{10} and the electron transport chain are ubiquitous within eukaryotic cells and rotenone is therefore a potent poison, being historically used as an insecticide, pesticide and piscicide.

M1. b. ii. Oligomycin (Sigma-Aldrich, St. Louis, MO, USA)

Oligomycin is a macrolide isolated from *Streptomyces diastatochromogenes* that acts as an electron transport chain inhibitor by inhibiting phosphorylation and abolishing ADP-stimulated respiration in intact mitochondria. Oligomycin binds to oligomycin sensitivity conferring protein in the F0 polypeptide (subunits 6 and 9) of ATP synthase, a proton pore located within the mitochondrial membrane; thereby blocking proton conductance and synthesis of mitochondrial ATP (Hundal et al., 1984).

M1. b. iii. Carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich, St. Louis, MO, USA)

FCCP is a small, amphipathic molecule that acts as a proton ionophore, forming a shield around transported protons and allowing the formation of a polar environment for the contained ion with a hydrophobic outer surface; and it is therefore capable of depolarizing both plasma membranes and mitochondrial membranes (To et al., 2010). Such activity is capable of resulting in uncoupling the link between the respiratory chain and phosphorylation systems within intact mitochondria, dissipating the proton gradient before it can be used for oxidative phosphorylation.

M1. b. iv. Angiotensin II (Alexis Biochemicals (Enzo Life Sciences), Exeter, UK; Cat. No. ALX-151-039-M005)

The biological characteristics of Angiotensin II are described earlier (see Introduction page 18 for more information).

M1. b. v. Losartan Potassium (Tocris Bioscience, Bristol, UK; Cat. No. 3798)

Losartan is a selective, competitive antagonist of the AT_1R. Systemically it antagonises vasoconstriction and release of aldosterone, thereby resulting in reduction in blood pressure through decreases in both total peripheral resistance and venous return. It has similar antagonistic effects at a paracrine and autocrine level. It is well absorbed orally and therefore commonly utilised for the treatment of hypertension (as with other RAS antagonists e.g. ACE-inhibitors) and renal disease secondary to diabetes and hypertension.

M1. b. vi. PD123319 ditrifluoroacetate ((6S)-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(2,2-diphenylacetyl)-6,7-dihydro-4H-imidazo[4,5-c]pyridine-6-carboxylic acid (Tocris Bioscience, Bristol, UK; Cat. No. 1361)

PD123319 is a potent, selective, non-peptide AT_2R antagonist. It may also have some antagonistic effects upon the action of Ang 1-7 (Walters et al., 2005, Bosnyak et al., 2011).

M1. b. vii. Histamine (Sigma-Aldrich, St. Louis, MO, USA)

Histamine is an organic, nitrogen-based compound with multiple functions, including a crucial role in immune responses. Its actions occur via binding with one of four histamine receptors (H1-4), all of which are G-protein coupled receptors, leading to an increase in intracellular calcium.

M1. b. viii. Antibodies

Primary and secondary antibodies to both RAS components and standard cellular components were sourced from a variety of manufacturers and utilized at manufacturers recommended concentrations (see Appendix Tables App2. a. and App2. b., pages 216-220 for further details).

M2. Cell-lines & Tissues

M2. a. HeLa Cells

A cell-line derived from cervical cancer cells, which contain a chromosome number of 82, with four copies of chromosome 12 and three copies of chromosomes 6, 8, and 17. The cells proliferate rapidly and contain a version of telomerase, which prevents the incremental shortening of telomeres during cell division, allowing them to circumvent the Hayflick Limit and achieve immortality (Ivankovic et al., 2007). HeLa cells contain no functioning angiotensin receptors [personal communication: Peter Varnai (Semmelweis Medical School, Budapest, Hungary)]. HeLa cells were kept in an incubator at 38 °C inside a 75 cm^2 flask containing Dulbecco's modified eagle's medium (DMEM (Cat. No.41966052); Invitrogen, Carlsbad, CA, USA), foetal bovine serum (FBS) and streptomycin.

M2. b. HepG2 Cells

An immortal cell-line derived from well-differentiated hepatocellular carcinoma cells, which contain 55 chromosomes. HepG2 cells display robust morphological and functional differentiation, rendering them a good model for the *in vitro* study of human hepatocytes.

HepG2 cells were kept in an incubator at 38 °C inside a 75 cm^2 flask containing Dulbecco's modified eagle's medium (DMEM (Cat. No.41966052); Invitrogen, Carlsbad, CA, USA), FBS and streptomycin.

M2. c. Rat Liver Tissue

Rat livers were surgically removed from Sprague-Dawley rats held at University College London (London, UK), where all Home Office regulations and codes regarding the housing and maintenance of the animals are strictly adhered to. Rats were humanely euthanised by placing them in a chamber within which the concentration of CO_2 was gradually increased.

The rats were subsequently dissected and the liver removed by surgical division of its anatomical attachments.

M2. d. Cell Culture

M2. d. i. Cell Passage

Cells were viewed under a light microscope to ensure 90–100 % cellular confluence within a flask. Spent medium was subsequently removed from the flask and the cells washed with phosphate buffered saline (PBS) and 1 ml of dilute trypsin (serine protease) solution added to the flask to hydrolyse and break attachments between the cells and the flask. The flask was then incubated at 38 °C for 3-5 minutes and the flask imaged again to ensure the cells have separated from the flask and are now suspended in solution. DMEM (6ml containing FBS and streptomycin) was added to the flask and rinsed over the separated cells. The subsequent mixture of DMEM containing cells in solution was collected by pipette and a third (2.3 ml of total 7 ml) of the solution added to three new 75cm^2 flasks, which were supplemented with a further 12 ml of medium before being labeled and placed back in an incubator at 38 °C.

M2. d. ii. Freezing Cells

A 2.3 ml solution of cells/medium was centrifuged at 250 g for four minutes at 4 °C and spent medium separated from the cellular pellet. The pellet was then resuspended in 500 µl of FBS:Dimethyl Sulfoxide (DMSO) (90 % : 10 %) solution in a cryotube wrapped in paraffin-coated film. The cryotube was labeled, placed in ice for half an hour and then transferred to a freezer at -20 °C.

M2. d. iii. Coverslips

Cell/medium solution (7 ml) from cell passage was further diluted with 5 ml of DMEM. The subsequent 12 ml of solution was gently agitated and 2 ml of solution added to each well of a 6-well plate, within which a 22 mm cover-slip had already been placed. The 6-well plate

was labeled and returned to an incubator at 38 °C to allow the cells to grow on the coverslip. Cover-slips were subsequently removed following adequate growth by removing spent medium with a pipette and extracting the cover-slip from the well utilising forceps.

M3. Cellular Sub-Fractionation & Mitochondrial Isolation

M3. a. Principle

Differential centrifugation and percoll density gradient separation is a method of separating cellular compartments (cellular sub-fractionation) based upon their density (low density components being cell membrane, mitochondrial associated membranes and endoplasmic reticulum; high density being mitochondria and nucleus), and was performed based upon standard methodologies (Sims and Anderson, 2008). Typically, sub-cellular fractionation performed on cells grown in flasks will yield smaller fractions than large pieces of tissues, rendering some of the sub-fractions isolated unsuitable for further experimentation because of the low protein concentration and volume.

M3. b. Sample Homogenisation

Freshly harvested livers from Sprague-Dawley rats or cells gathered from flasks immediately following trypsin isolation were stored in cold PBS, on ice, and kept on ice throughout the mitochondrial isolation process (minimum 4° C). Rat liver samples were initially homogenized by cutting into small pieces/mincing with scissors, while repeatedly washing with PBS until the supernatant was clear and blood-free (to prevent sample contamination by blood cells). The cell or tissue samples were mixed with Solution A (1 ml per gram of tissue) and Protease Inhibitor (Protease Inhibitor cocktail P8340; Sigma-Aldrich, St. Louis, MI, USA) added (1 µl per gram of tissue). Samples were further homogenised utilising a Dounse homogeniser (at least 40 runs total: 20 runs with loose-fitting Teflon pestle and 20 runs with tight-fitting Teflon pestle) and a drill-powered Potter-Elvehjem homogeniser (at least ten runs). The resultant homogenate (H) was aliquoted into 2 ml samples and was either stored at -20 ^{o}C in cryotubes or immediately underwent differential centrifugation.

M3. c. Differential Centrifugation (See Figure M1)

The homogenate (H) was centrifuged at 800 times gravity (g) at 4 °C for ten minutes in a Beckman J2-MC centrifuge (with JA-20 rotor) to yield a nuclear pellet (N) and a postnuclear supernatant (PNS). N was subsequently resuspended in solution A.

PNS was spun at 10,300 g for ten minutes at 4 °C in a Beckman J2-MC centrifuge (with a JA-20 rotor) to yield a crude mitochondrial pellet (CM) and a postmitochondrial supernatant (PMS).

PMS was centrifuged at 25,000 g for 30 minutes at 4 °C in a Beckman J2-MC centrifuge (with a JA20 rotor) to yield an enriched lysosomal pellet (L) and a postlysosomal supernatant (PLS).

PLS was centrifuged at 100,000 g for one hour at 4 °C in a Beckman 70 Ti rotor centrifuge to yield a microsomal pellet (MS) and a supernatant enriched with cytosolic proteins (C). MS was resuspended in Solution A. All samples under investigation (H, C, N, L, MS, CM, PM, MAM) were subsequently aliquoted and stored at -20 C.

M3. d. Percoll Density Gradient Separation

CM was gently layered on top of Solution B and 30 % Percoll (by volume) in a 10 ml polycarbonate ultracentrifuge tube and centrifuged at 95,000 g for 30 minutes at 4 °C in a Beckman L-70 Ultra-Centrifuge (with SW41 T rotor) to yield two fractions: an upper diffuse white band (mitochondria associated membranes; MAM) and a lower, denser band containing purified mitochondria (PM) (see Figure M2).

PM was removed carefully, diluted with Solution A and washed twice by centrifuging at 6300 g for ten minutes to remove the Percoll, and then resuspended in Solution A and stored at -20 °C.

The MAM fraction was carefully removed and washed by centrifuging in Solution A at 6300 g for ten minutes at 4 °C. The supernatant was further centrifuged at 100,000 g for one hour at 4 °C in a Beckman 70 Ti rotor and the pellet resuspended in Solution A.

The various pellets subsequently underwent protein quantification.

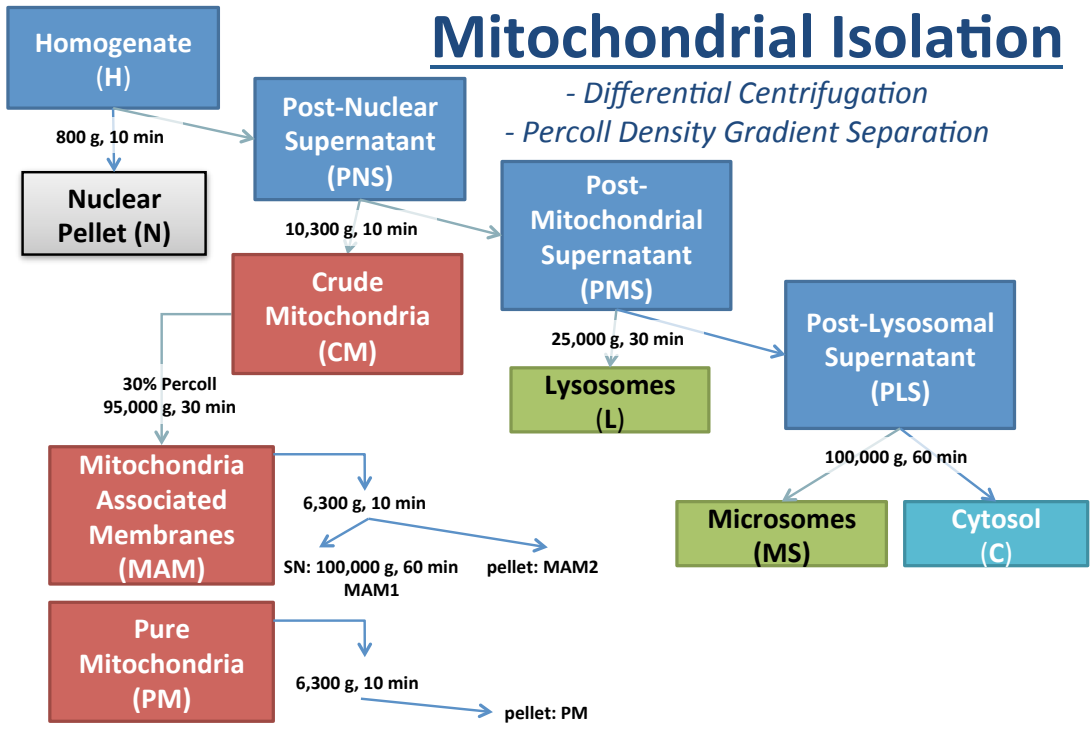

Figure M1. Summary of mitochondrial isolation by differential centrifugation and percoll density gradient separation.

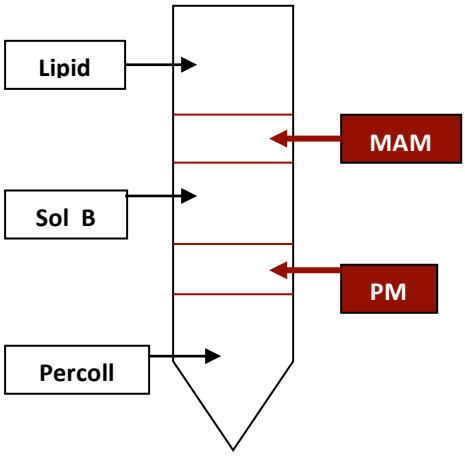

Figure M2. Schematic diagram of mitochondria-associated membranes (MAM) and pure mitochondria (PM) layers isolated following cellular sub-fractionation by differential centrifugation and percoll density gradient separation.

M3. e. Protein Quantification

The following process, utilizing a BCA (Bicinchoninic acid) protein assay kit, allowed colourimetric detection and quantification of total protein concentrations by relying on the chelation of copper with peptides/proteins containing three or more amino acid residues, within an alkaline environment (containing sodium potassium tartrate), to form a light blue complex through the production of reduced Cu^{1+} from Cu^{2+} (Biuret reaction). Cu^{1+} is subsequently detected by chelation of two BCA molecules with one Cu^{1+} ion, in a highly sensitive and selective fashion, producing a water-soluble, purple solution, which exhibited a strong linear absorbance at 562 nm with increasing protein concentrations. The absorbance was then compared to samples containing standard concentrations of common proteins, such as BSA, which have been prepared alongside the sample under analysis. Protein quantification was performed utilising a BCA protein assay kit (Thermo Scientific (Cat. No. #23225); Waltham, MA, USA) and standard methodology (for full protocol see http://www.piercenet.com/instructions/2161296.pdf).

A set of protein solutions containing set concentrations were created, utilising the BSA ampoules contained within the protein assay kit, to produce a range of diluted protein standards within a working range of 20-2000 µg/ml protein concentration. The working reagent containing Cu^{2+} ions and an alkaline environment was also made up in preparation for the reaction. The samples under investigation were also pipetted into 25 µl aliquots, ready for analysis. Each standard or sample (25 ul) was subsequently placed into a well in a 32-well microplate. Working reagent (200 ul) was added to each well and the solution placed on a shaker for 30 seconds, before incubation at 37 °C for 30 minutes. Absorbance was subsequently measured at 562 nm on a plate reader. The average 562 nm absorbance of the blank standards prepared (containing no protein) was subtracted from all other standards and samples. A standard curve was subsequently plotted of the corrected 562 nm measurement of each BSA standard (y axis) against its concentration in µg/ml (x axis). This standardised curve was subsequently utilised to determine the protein concentrations of all unknown samples.

M4. Western Blotting

M4. a. Principle

Western blotting is a process by which proteins are separated by polyacrylamide gel electrophoresis, before being transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. The membrane is subsequently imaged by applying a secondary antibody linked to a reporter enzyme, usually horseradish peroxidase, which chemiluminesces when exposed to light revealing protein 'bands' on the surface of the membrane. Optical density analysis is subsequently utilised to determine the relative amount of protein present in each band and band size approximations are made by comparing the stained bands to the marker loaded at the time of electrophoresis.

M4. b. Polyacrylamide Gel Electrophoresis

Samples derived from cellular sub-fractionation were separated by polyacrylamide gel electrophoresis utilising standard techniques, following protein quantification (See http://tools.invitrogen.com/content/sfs/manuals/blotmod_pro.pdf and

http://tools.invitrogen.com/content/sfs/manuals/surelock_man.pdf for full protocol). Samples were initially diluted into 30 µl aliquots containing 30 µg of protein under investigation per sample (as per protein quantification results). The separate sample aliquots were subsequently pipetted into wells in a Bis-Tris gel (NuPAGE® Novex 4-12% Bis-Tris gel (1.5 mm, 10 well) (Invitrogen (Cat. No. NP0335BOX); Carlsbad, CA, USA)), with 6-8 µl of protein size standards (Precision Plus protein standards (Bio Rad Laboratories (Cat. No. 161-0374); Hercules, CA, USA)) pipetted into the final 10th well. The gel was placed into an XCell II™ SureLock Mini Cell apparatus (Invitrogen (Cat. No. E19051); Carlsbad, CA, USA) containing running buffer (MOPS SDS running buffer (20x) (Invitrogen (Cat. No. NP0001); Carlsbad, CA, USA)) diluted with distilled water and 200 V applied for 45-60 minutes (see Figure M3).

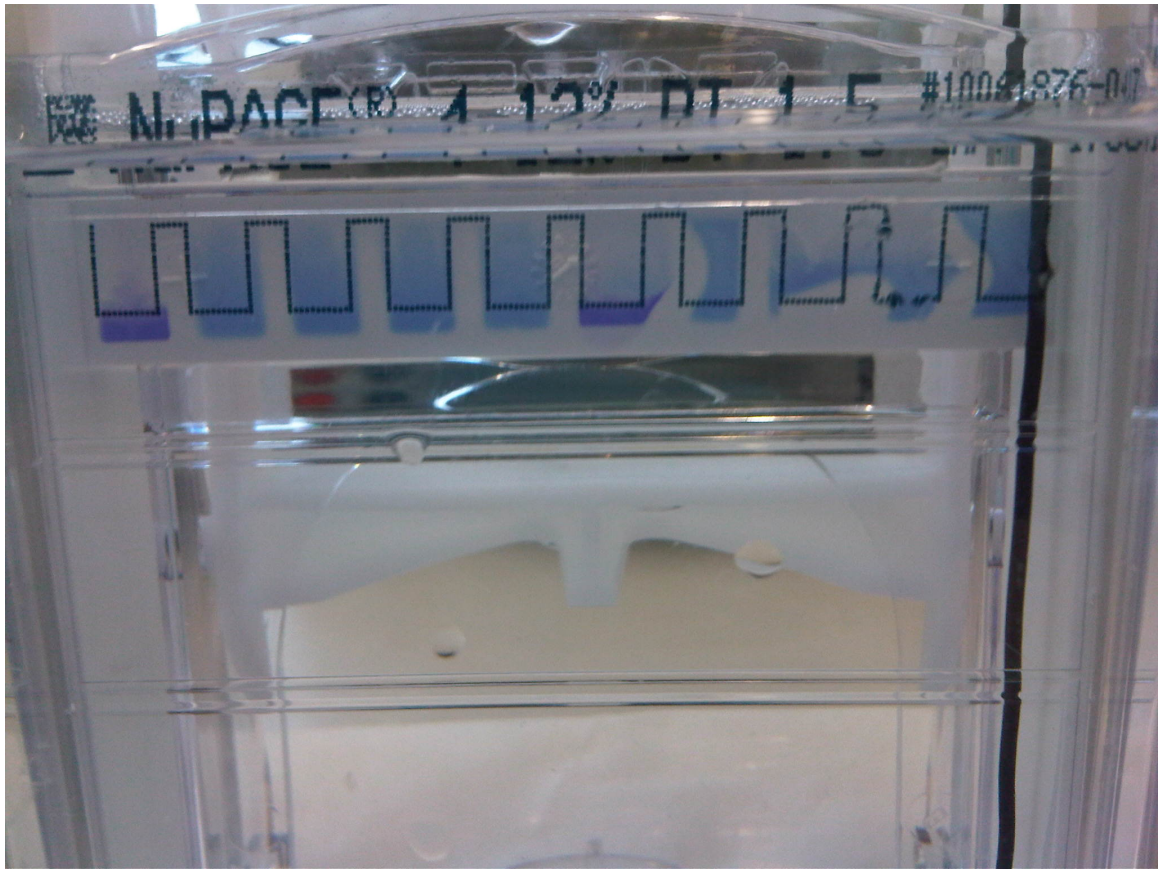

Figure M3. Image of polyacrylamide gel electrophoresis utilising protein samples in a 4-12 % Bis-Tris gel. Blue wells contain 30 µl of solution containing 30 µg of protein sample; whereas, purple wells contain 6-8 µl of protein size standards.

M4. c. Transfer to PVDF Membrane

Once electrophoresis was complete, the gel was removed from its cassette utilising a palette knife and placed in a sandwich setup within an electro-transfer apparatus (XCell II™ Blot Module apparatus, Invitrogen (Cat. No. E19051); Carlsbad, CA, USA) for transfer to a PVDF membrane (Immobilon-P transfer membrane, Millipore (Cat. No. IPVH00010); Billerica, MA, USA). The chamber was filled with transfer buffer (50 ml of NuPage transfer buffer (Invitrogen (Cat. No. SKU#NP0006)), methanol (100 ml) and distilled water (850 ml) and 10 V applied continuously overnight at 4 °C.

M4. d. PVDF Membrane Antibody Staining

The PVDF membrane was removed from the sandwich setup and placed in a glass jar. Non-specific membrane-binding was prevented ('blocking') by soaking the membrane in PBS containing 5 % bovine serum albumin (BSA; Albumin from bovine serum (pH 5.4), Sigma-Aldrich (Cat. No. A8022); St. Louis, MI, USA) solution containing a low percentage of Tween20 detergent for two hours at room temperature, while gently agitating on a rocker. Following blocking, a dilute PBS solution of primary antibody, BSA (2.5 %) and Tween20 (0.1 %) was incubated with the membrane under gentle agitation for one hour at room temperature (specific antibody concentrations varied depending upon antibody utilised- see Appendix Table APP2. a. and APP2. b.). Unbound primary antibody was removed by subsequently rinsing the membrane in a low concentration of PBS/Tween20 (0.1 %) solution twice for 30 minutes each, and the membrane then exposed to a a dilute PBS solution containing secondary antibody and BSA (2.5 %)/Tween20 (0.1 %) solution linked to a reporter enzyme (usually horseradish peroxidase) for one hour at room temperature, while gently agitating. The membrane was again rinsed in low concentration Tween20 solution twice for 30 minutes at room temperature to remove all excess antibody.

M4. e. Membrane Imaging

Immunoreactive bands were detected by placing the membrane on an acetate sheet and chemiluminescent detection reagents added (ECL Plus Western Blotting Detection Reagents (Cat. No. RPN2132), GE Healthcare/Amersham, Little Chalfont, UK). Excess solution was removed utilising absorbent filter paper and the membrane placed in a chamber (Chemidoc MP system, BioRad, Hercules, CA, USA) for antibody detection by chemiluminescence.

M5. Immunofluorescence

M5. a. Principle

The basic principle of immunofluorescence depends upon selectively exciting fluorescent molecules applied to cells or tissue sections, in such a way that each fluorescent molecule exists as a separate light source. When a molecule of fluorescent dye absorbs a quantum of light of sufficient energy, an electron is elevated to a higher energy level thus forming an excited state. This electron will eventually return to its previous 'ground' state, and when it does it may emit a quantum of light. In general less energy is emitted than was adsorbed and therefore the wavelength of fluorescent light emitted is no longer the same as that of the initial light adsorbed. Thus fluorescence microscopy is useful in that not only is it extremely sensitive, but it is also highly specific. Fluorescence microscopy can also allow the analysis of the molecular properties and environment of the fluorescent molecules.

Confocal microscopy is a technique that allows the analysis of light focused at an extremely narrow band within a specimen. Thus it allows the user to gather depth-selective information on the three-dimensional structure of a microscopic object, without mechanical sectioning and without confusion that can be caused by images derived from the areas above and below the section. The confocal microscope employs an illuminating laser light system that is very strongly convergent, producing a very shallow scanning spot in the object plane. The light or fluorescence reflected back from this spot is in turn directed to a photomultiplier through a detector pinhole. The light is analysed here and displayed by a computer as a pixel on a screen. In order to gain a complete image of the object, a system of mirrors is used to move the light point over the specimen, illuminating a single spot at a time. The information from each individual spot is recorded and stored in the computer, and the image is simultaneously displayed on a high-resolution video monitor to create a visual image. The conjugation of the detector pinhole to the illumination pinhole ensures that it is only information from the focal plane that reaches the detector. This accounts for the confocal microscope's unique ability to create images of sections through a sample, and the user can literally dissect layer by layer through the entire thickness of the specimen. The out-of-focus regions are also subtracted from the image by the computer programme, thus enhancing its sharpness.

M5. b. Cell Fixation & Permeabilisation

Cells were grown on cover-slips, within a 6-well plate, in an incubator at 38 °C. When cover-slips were approximately two-thirds covered in confluent cells, spent medium was removed and the cover-slips were twice washed briefly with PBS (2 ml per well) at room temperature. The cells were subsequently fixed by incubating the cover-slips with 4 % paraformaldehyde for five minutes at room temperature (1 ml per well), and then twice washed at room temperature in PBS (2 ml per well), while gently agitating on a rocker. For further fixation, pre-cooled acetone (-20 °C; 1 ml per well) was added to the cover slips, which were left for ten minutes at -20 °C and then twice washed at room temperature in PBS (2 ml per well), while gently agitating on a rocker. The cells were subsequently permeabilised by incubating cover-slips twice with 0.025 % Triton X-100 for five minutes at room temperature (1 ml per well), before washing twice with PBS (2 ml per well). To prevent non-specific antibody binding, the cover-slips were rested in blocking solution (10 % donkey serum in PBS; Donkey Serum, Sigma-Aldrich (Cat. No. D9663); St Louis MI, USA) for 30 minutes at 4 °C, and then washed in 1 % donkey serum in PBS for five minutes.

M5. c. Antibody Staining

Following fixation and permeabilisation, the cover-slips were incubated for 18 hours (or overnight) at 4 °C with the primary antibody solution (primary antibody in 1 % donkey serum/PBS solution) in a sandwich setup (see Figure M4). The concentration of primary antibody varied according to the specific antibody utilised (for further information see Appendix Tables App2. a. and APP2. b., pages 216-220).

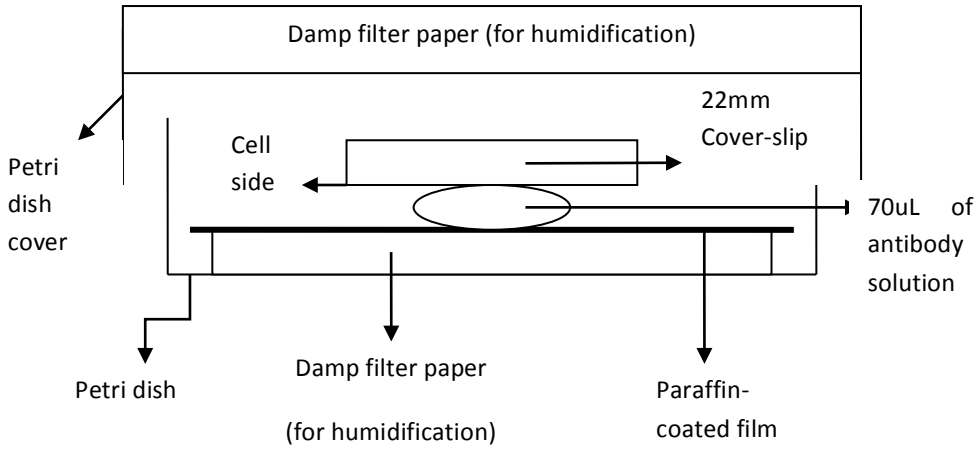

Figure M4. Schematic diagram of sandwich setup for antibody staining and immunofluorescence.

The cells were subsequently washed in PBS and 0.025 % Triton X-100 three times for five minutes each, while gently agitating on a rocker at room temperature. They were then incubated with the secondary antibody solution (secondary antibody in 1 % donkey serum/PBS solution) in a sandwich setup (see Figure M4) for one hour at room temperature in the absence of light. The concentration of secondary antibody applied varied according to the specific antibody utilised (see Appendix Tables App2. a. and APP2. b., pages 216-220 for further information). The cells were washed again (PBS; two times for ten minutes, while agitating, in the absence of light) and then incubated with Hoescht 33258 (1 (Antibody) : 1000 (PBS); vol : vol) for one minute in a 6-well plate (1 ml per well), in the absence of light. The cells were washed again in PBS for ten minutes in the absence of light, the slides dabbed dry with filter paper and placed cell-side down onto a glass slide. The edges were sealed with nail varnish, protected from light by wrapping in foil and imaged.

M5. d. Confocal Microscopy of Stained Cells

Immunofluorescence was performed on a Zeiss LSM 510 META confocal microscope (Carl Zeiss; Oberkochen, Germany).

M6. Functional Fluorescence Microscopy

M6. a. Principle

Functional fluorescence microscopy refers to the process of imaging live cells on a confocal microscope to assess how the addition of certain substrates alters cellular processes in real-time. It relies upon the initial addition of various fluorescent molecules to the live cell, which are detected at specific wavelengths, the intensity of which alter as the metabolic process under examination changes; thus allowing quantitative study of molecular interactions within live cells (for further information see 'M1. Materials'; page 61). Utilising this technique it is possible to measure real-time changes in cellular calcium and NADH concentration, and mitochondrial membrane potential, as well as various other cellular processes.

M6. b. Preparation of Cover-Slips

Live cells (HeLa or HepG2) were grown on 22 mm cover-slips in an incubator at 38 °C (for further information see 'M2. Cell Culture'; page 66). When the cells reached 50-60 % confluent growth on the cover-slip, 25 µl TMRM and 1 µl Fluo-4 were added to the medium solution and the cover-slips again left at 38 °C in an incubator for 30 minutes. Finally, the cover-slips were washed with PBS three times to remove all excess dye.

M6. c. Functional Microscopy

The cover-slips were placed on a heated stage at 38 °C in a confocal microscope with UV laser capabilities (Zeiss LSM 510 META confocal microscope (Carl Zeiss; Oberkochen, Germany)) and left to equilibrate for 10 minutes prior to further assessment. A UV laser at 543 nm was applied to the live cells to measure mitochondrial membrane potential, as extrapolated from TMRM fluorescence; cytosolic calcium was measured via excitation of Fluo-4 at 488 nm; and NADH concentration detected via autofluorescence at 364 nm.

A baseline measurement was taken for five minutes and various solutions added to the cover-slip with a pipette, to assess effects upon excitation at the various wavelengths under investigation. Solutions were added at 1 μM concentration and included angiotensin II, histamine, FCCP and rotenone (for further information see 'M1. Materials'; page 61).

M6. d. Results Analysis

Intensity of fluorescence at the various wavelengths was assessed utilising pre-installed optical density software. Alterations in fluorescence between baseline and post-reagent addition, within a whole field under investigation, were subsequently quantified and calculated.

M7. Immunogold Labeling & Electron Microscopy

M7. a. Principle

Transmission electron microscopes (EM) employ an electron beam emitted from an electron source, usually a tungsten filament cathode, which is controlled and focused by electrostatic and electromagnetic lenses, to illuminate a specimen and produce a magnified image; whereas, scanning EM produces images by scanning over a sample with a high energy focused beam of electrons, which interact with electrons within the sample, producing secondary electrons, transmitted electrons and back-scattered electrons, as well as X-rays, light and current. Electrons have wavelengths 100,000 x shorter than visible light (photons) and electron microscopes therefore achieve much greater resolution than light-powered optical microscopes, potentially achieving magnifications of up to 10,000,000 x. Biological specimens for EM analysis are fixed, dehydrated and embedded within polymer resin, to allow thin sections to be prepared for analysis.

M7. b. LR White processing

Rat liver samples were dissected into small pieces with scissors/scalpel and fixed for 8-12 hours (or overnight) in 4 % paraformaldehyde / 0.5 % glutaraldehyde (in 0.1 M cocadylate buffer at pH 7.4). Samples were subsequently washed in 4 % sucrose (in 0.1 M cocadylate (pH 7.4)) three times and stored at 4 ºC before being dehydrated in graded concentrations of ethanol (50 %; 70 %; 80 %; 85 %; and 95 %) for 60 minutes each, followed by three separate 30 minute exposures to 100 % ethanol.

Samples were then infiltrated at room temperature with LR White low viscosity acrylic embedding medium (a polyhydroxy-aromatic, hydrophilic acrylic resin with low toxicity, ultra-low viscosity and low miscibility with water and gently shaken on a rotor for three separate four hour periods in graded solutions of LR White : 100 % ethanol solution (ratios of 1 : 1, 2 : 1 and 3 : 1) before being left in pure LR White for two 12-hour periods. Samples were then embedded and polymerized at 60 ºC for 20-24 hours.

M7. c. Immunogold Labeling

Embedded tissue was subsequently cut into ultra-thin sections and mounted on formvar-coated grids and allowed to dry. Once dried, the grids were washed with 0.05 M Tris-buffered saline (TBS) containing 1 % bovine serum albumin (BSA; Albumin from bovine serum (pH 5.4; Sigma-Aldrich, St Louis, MI, USA)) for 10 minutes at room temperature and sections were then incubated overnight with the primary antibody of choice (in 0.05 M TBS with 1 % BSA at 4º C).

The grids were then washed in 0.05 M TBS with 0.05 % Tween-20 three times and incubated for 1 hour at room temperature with the secondary antibody of choice (in 0.05 M TBS with 1 % BSA at 4 ºC).

Finally, the grids were washed in 0.05 M TBS with 0.05 % Tween-20 three times (each 5 min length), and again in double-distilled water, before being allowed to dry. Sections were then counterstained with 4 % uranyl acetate for 20 min, followed by lead citrate for 5 minutes, both at room temperature.

M7. d. Electron Microscopy

Fresh rat liver samples were delivered on ice to the electron microscopy unit at the Royal Free Hospital (Electron Microscopy Unit, UCL Medical School, Royal Free Campus, Rowland Hill Street, Hampstead, London NW3 2PF), where sample preparation by dehydration, fixation and embedding, as well as all electron microscopy, was performed by experienced EM operators.

M8. Immunoprecipitation

M8. a. Principle

Crosslink immunoprecipitation refers to the process of antigen purification utilising a specific antibody. The principle involves crosslinking to covalently attach a specific antibody to a protein A/G agarose resin (recombinant protein combining antibody-binding domains from protein A and G), which is then incubated with a protein mixture containing the antigen, allowing the antibody : antigen complex to form. After washing to remove unbound, undesired sample components, the antibody : antigen complex, immobilised by crosslinking with disuccinmidyl suberate (DSS) on beaded agarose resin, is eluted and separated by centrifugation (see Figure M5).

Samples under investigation were immunoprecipitated utilising a Pierce Crosslink IP kit (Thermo Scientific (Cat. No.#26147); Waltham, MA, USA), with all steps carried out at 4 °C (unless otherwise stated) and all centrifugations at low speed (1000-3000 g) for 30-60 seconds (see http://www.piercenet.com/instructions/2162134.pdf for full protocol).

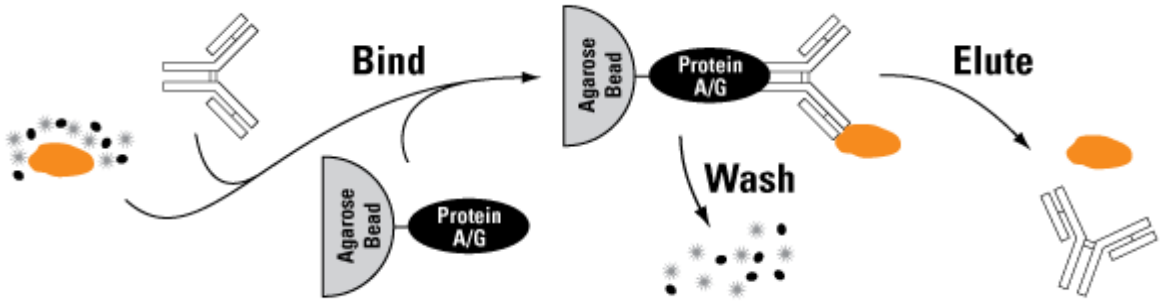

Figure M5. A schematic diagram demonstrating crosslink immunoprecipitation. Figure taken from Piercenet (see http://www.piercenet.com/browse.cfm?fldID=FE0FBC6F-61BB-41DF-AD2E-008247C95177).

M8. b. Binding of antibody with Protein A/G Agarose Resin

A solution containing 10 µg of antibody was prepared through dilution of the antibody with coupling buffer and ultrapure water and then added to 20 µl of Pierce Protein A/G Plus Agarose resin and mixed on a rotor for 30-60 minutes; followed by washing via centrifugation with coupling buffer.

M8. c. Crosslinking of bound antibody

The prepared antibody : resin complex was incubated with DSS, ultrapure water and coupling buffer and placed on a mixer for 30-60 minutes at room temperature. The solution was washed in Elution buffer and IP / Lysis Wash buffer via centrifugation, prior to proceeding to immunoprecipitation.

M8. d. Immunoprecipitation

The sample under investigation was diluted with IP / Lysis Wash buffer to create a volume of 300-600 µl containing 500-1000 µg of total protein. This was added to the prepared antibody : resin complex and incubated overnight on a rotor at 4 °C.

M8. e. Antigen Elution

The mixture was washed via centrifugation with IP / Lysis Wash buffer and Conditioning buffer and 5 ul of 1 M Tris (pH 9.5) added to neutralise the low pH and allow functional assays. The sample was incubated and centrifuged with elution buffer and the eluate collected for further analysis or stored at -20 °C.

M9. Mass Spectrometry

M9. a. Principle

Mass spectrometers are analytical tools that measure the molecular mass of samples applied to them with an exceedingly high degree of accuracy that allows both quantitative and qualitative uses. Mass spectrometry can be used to quantify the amount of a compound or molecule in a sample, detect even minor changes in mass (such as the alteration of one amino acid), the molecular formula of organic compounds, or a compound's structural information, by observing its fragmentation.

Mass spectrometers contain three fundamental parts: an ion source, which can convert gas phase sample molecules into ions or move ions in solution into the gas phase; a mass analyser, which is capable of sorting ions by their mass via the application of electromagnetic fields; and a detector, which assesses the abundance of each ion present. All three parts are maintained within a vacuum to allow the ionised molecules to travel from one end of the machinery to the other without the hindrance of air molecules and the whole system is controlled by computer software. Heavier ions travel more slowly across the machinery and the subsequent mass : charge ratio (m / z spectrum) is compared to a database. Often a sequence tag of only 4-5 amino acids can be utilised to identify a protein or peptide from a database.

The sample under investigation is introduced into the ionisation source, where its molecules become ionised. The ions are subsequently separated by the analyser according to their mass to charge ratio and the separated ions are subsequently detected in the detector and their frequency also assessed for subsequent presentation in the form of an m/z spectrum.

MALDI–TOF (Matrix-Assisted Laser Desorption Ionisation–Time Of Flight) (Hillenkamp et al., 1991) is a type of mass spectrometer that is particularly useful in the assessment of thermolabile, non-volatile organic compounds of high molecular mass such as proteins, peptides and glycoproteins. MALDI involves pre-mixing the sample with a highly-absorbent matrix compound in a volatile solvent, which is capable of converting laser light into excitation energy, with subsequent sample bombardment by laser light (usually pulsed

nitrogen laser of 337 nm wavelength) to produce ionisation. Proteomic analysis can be performed utilising this technique by excising bands or spots from a gel, enzymatically digesting the peptides or proteins within each band or spot and then analysing the resultant mixture via MALDI-TOF. The result can be compared to a database of known peptides/proteins, where it is either identified as a known protein, or not identified because it is previously uncharacterised, or the result does not provide enough information to distinguish it between a few potential results and further information is required, either through increasing the concentration of the molecule or cleaning of the sample.

M9. b. Sample Preparation for Mass Spectrometry

Samples under investigation underwent gel electrophoresis on a 4-12 % Bis-Tris gel, prior to silver staining and subsequent excision of protein bands of interest. The bands subsequently underwent reduction and alkylation, prior to deglycosylation and trypsin digestion prior to proteomic analysis via mass spectrometry.

M9. b. i. Silver Staining

(see http://tools.invitrogen.com/content/sfs/manuals/silverquest_man.pdf for full protocol)

Silver staining was performed utilising the SilverQuest^{TM} Silver Staining Kit For Mass Spectrometry (Compatible Silver Staining of Proteins in Polyacrylamide Gels by Invitrogen, Carlsbad, CA, USA; Catalogue Number LC6070). Silver staining is based upon the chemical reduction of silver ions to metallic silver on a protein band and is a sensitive method of detecting most proteins, capable of picking up protein concentrations at sub-nanogram levels and is 30-fold more sensitive than coomassie staining (Rabilloud et al., 1994). Ultrapure water was used throughout and all incubations were performed on a rotary shaker at a speed of 1 revolution/second at room temperature.

After gel electrophoresis, the gel was removed from the cassette and placed in a glass jar, where it was rinsed with ultrapure water. The gel was subsequently fixed in 100 ml of fixative for 20 minutes. The fixative was subsequently removed and the gel washed in 30 % ethanol before that was also removed and 100 ml of sensitizing solution added for 10

minutes. The sensitizing solution was removed and the gel washed again in 30 % ethanol for 10 minutes and then in 100 ml of ultrapure water for 10 minutes before being incubated in 100 ml of staining solution for 15 minutes. The staining solution was subsequently removed and the gel washed with ultrapure water for one minute before being incubated in 100 ml of developing solution for 4-8 minutes until the desired band intensity was achieved, at which point 10 ml of stopper was added to the gel still immersed in developing solution and the reaction incubated for 10 minutes. When the solution colour changed from pink to colourless (indicating that the development reaction has ceased) all solution was removed from the container and the gel washed in ultrapure water for 10 minutes.

M9. b. ii. Deglycosylation (For full protocol see:

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/pp0201bul.Par.0001.File.tmp/pp0201bul.pdf)

Deglycosylation was performed using the Glycoprofile II Enzymatic In-Solution N-Deglycosylation Kit (Sigma Aldrich, St. Louis, MI, USA; Cat No. PPO201). Glycosylation is a common post-translational modification of proteins in eukaryotic cells, where such glycoproteins are crucial for a number of biological functions. Human glycoproteins contain one of three major types of oligosaccharide or glycan: N-linked, O-linked or glycosylphosphatidylinositol lipid anchors, although variations in glycan structure and degree of saturation of available glycosylation sites often results in considerable heterogeneity in the mass and charge of glycoproteins. Removing a class of glycan aids in the study of glycoproteins and removal of N-linked glycans in particular reduces the heterogeneity of both mass and charge to assist in analysis by mass spectrometry. N-linked glycans link to the protein backbone via amide bonds to asparagine residues (whereas, O-linked attach to the hydroxyl group of serine or threonine), which can be broken by enzymatic degradation utilizing peptide N-glycosidase F (PNGase F). The ensuing peptide is left intact apart from the deamination of asparagine to aspartic acid at the site of the attachment.

The RNase B Standard (90 µl of 1.1 mg / ml) was added to a siliconised Eppendorf tube and 5 µl of the denaturant Solution (2 % octyl β-D-glucopyranoside with 100 mM 2-

mercaptoethanol) added and mixed briefly before incubation at 100 °C for 10 minutes. The solution was allowed to cool to room temperature and gently spun briefly before 5 µl of (1 X) Reaction Buffer was added and the solution was mixed and spun again briefly (glycoprotein concentration now 1 mg / ml). The solution was separated into two 50 µl aliquots, one to act as a control and the other as the working mixture. Then 5 µl of PNGase F Enzyme Solution (500 units / ml; 2.5 units of enzyme) was added to the working solution and 5 µl of ultrapure water to the control sample, and the solutions spun briefly before incubating at 37 °C overnight with 1000 µg of protein sample. The reaction was stopped by heating the solution at 100 °C for 10 minutes, before the solution was allowed to cool to room temperature and spun briefly in preparation for analysis.

M9. b. iii. Trypsin Digestion

(for full protocol see

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/pp0100bul.Par.0001.File.tmp/pp0100bul.pdf)

Protein bands of interest were digested by the serine protease, trypsin, which hydrolyses peptide bonds at the carboxyl side of arginine and lysine residues, resulting in a solution containing highly specific cleavage sites and peptide fragments. Trypsin digestion was performed using the Trypsin Profile IGD Kit (Sigma Aldrich, St. Louis, MI, USA; Cat No. PPO100) and standard methodologies (Speicher et al., 2000, Sechi and Chait, 1998).

The band of interest was excised from a gel, using a razor blade, and placed in a siliconised eppendorf tube using tweezers. The gel piece underwent destaining through twice adding 200 µl of Destaining Solution and incubating at 37 °C for 30 minutes. Each time, the resulting solution was removed and discarded. The gel piece was subsequently dried in a centrifugal evaporator (Savant Speed Vac; Thermoscientific, Waltham, MA, USA) for 30 minutes. 20 µl of trypsin solution (containing 0.4 ug of trypsin) and 50 µl of the Trypsin Reaction Buffer was subsequently added to the gel piece and the solution incubated overnight at 37 °C. The resultant solution, containing the extracted peptides was removed from the eppendorf tube and transferred to a new siliconised eppendorf tube and delivered for mass spectrometry as soon as possible.

M9. c. Mass Spectrometry

Mass spectrometry was performed on a dual-source (MALDI and ESI (Electrospray Ionisation)) time-of-flight mass spectrometer, by experienced technical staff, at one of two centres: Institute of Child Health, University College London, London, UK or Taplin Mass Spectrometry Facility, Harvard Medical School, Boston, USA.

M10. Oxygen Consumption

M10. a. Principle

Clark electrodes measure oxygen on a catalytic platinum surface. The electrode is isolated from the reaction chamber (containing cells or isolated mitochondria) by a thin membrane permeable to molecular oxygen and allows oxygen to reach the cathode, where it is electrolytically reduced. This electrolytic reduction allows a current to flow, which is proportional to the O_2 in the solution, provided that the solution is constantly stirred/mixed.

M10. b. Sample Preparation

Live cells (HeLa or HepG2) were grown in culture flasks in medium at 38 °C. A dilute trypsin solution was added to the cells in the flask to separate them and the cells in suspension placed in a plastic culture tube in ice. Cells in 50 µl of the solution were subsequently manually counted under a light microscope to provide a representative number for the whole solution and, following appropriate calculation, 2 million cells were added to 200 µl KRB and the solution gently mixed to provide an even suspension.

M10. c. Measurement of Mitochondrial Oxygen Consumption

The solution containing KRB and 2 million cells was placed within the chamber with a magnetic stirrer and allowed to equilibrate at 38 °C for five minutes. The rate of oxygen consumption was then set to 100 %, representing the baseline rate of oxygen consumption utilizing endogenous substrates and ADP. The baseline rate was subsequently measured for two minutes. Various reagent solutions (1 µM solutions) were subsequently added by pipette, via a hole in the chamber lid, including angiotensin II, histamine, oligomycin and FCCP, and the rate of oxygen consumption again measured for two minutes after addition of each reagent. The average rate of oxygen consumption was subsequently calculated by pre-installed computer software (LabChart 7 Reader; Dunedin, New Zealand) and expressed as a change in oxygen consumption per minute, compared to a basal level of 100 % (therefore 0.02 equals decrease in oxygen consumption of 2 % per minute from basal rate).

RESULTS 1:

PRESENCE OF RAS COMPONENTS IN HEPATIC MITOCHONDRIA

Abstract

Introduction: Individual RAS components have previously been shown to be associated with mitochondria. However, such studies are few, address a limited range of cells, have often relied upon immunohistochemistry alone to demonstrate RAS components, have rarely sought to identify the co-existence of multiple RAS components, and have failed to seek the presence of some entirely. Therefore, which RAS components are found within mitochondria and their precise intra-mitochondrial location remains unclear.

Methods: RAS components in the mitochondria of rat liver cells and HepG2 cells were investigated via immunogold labeling and electron microscopy, immunofluorescence and confocal microscopy, and Western blotting.

Results: Western blotting demonstrated renin, AT_1R, AT_2R and angiotensin within sub-fractionated mitochondrial compartments in both rat liver tissue and HepG2 cells; immunofluorescence and confocal microscopy of HepG2 cells demonstrated the presence of all classical RAS components within HepG2 cells, with renin, ang and ACE co-localising with mitochondria; and immunogold labelling and electron microscopy demonstrated ACE, ang, AT_1R and AT_2R in association with rat liver mitochondria and mitochondria of the HepG2 cell line. ACE, at its usual size of 170-180 kDa was absent from all mitochondrial sub-fractions in both rat liver tissue and HepG2 cells; however, there was enrichment of a 55 kDa molecule, when stained with ACE C-terminal antibodies.

Discussion: Taken together, the data presented in this chapter suggest the possibility of certain RAS components being present within mitochondria; however, the results are inconsistent between techniques and therefore cannot provide unequivocal evidence for the presence of a mitochondrial RAS. The unexpected finding of a 55 kDa molecule stained with ACE (C-terminal) antibody may potentially represent a novel, truncated form of ACE.

R1.1 Introduction

Individual RAS components have been shown to be associated with mitochondria (for full discussion see 'I3: RAS & Mitochondria'; page 30). However, such studies are few, address a limited range of cells, have often relied upon immunohistochemistry alone to demonstrate RAS components, have rarely sought to identify the co-existence of multiple RAS components, and have failed to seek the presence of some entirely. Indeed, the reliance on one antibody in each of the above studies renders the authors interpretations open to significant criticism. Further, results may be explained by non-specific binding (e.g. Erdmann et al., 1996) and criticised for the lack of consistency in the authors' choice of cells and tissues (e.g. Abadir et al., 2011). Therefore, which RAS components are found within mitochondria and their precise intra-mitochondrial location requires further elucidation.

In an attempt to circumvent the above issues, the presence of all major RAS components were sought in the mitochondria of rat liver cells and HepG2 cells using immunogold labeling and electron microscopy, immunofluorescence and confocal microscopy, and Western blotting. Thus, a variety of techniques, as well as tissues and cell-lines, were utilised to provide an overall, robust assessment of whether RAS components were actually present within hepatic mitochondria. Both tissues and cell-lines were utilised to overcome the drawbacks of using one or other alone. Liver cell lines and tissue were utilised (as opposed to tissue or cell-lines derived from other body areas) due to the high density of mitochondria within hepatocytes and the subsequent high mitochondrial yield on subcellular fractionation.

R1.2 Methods

R1.2.a. Western Blotting

Western blotting was performed following subcellular fractionation and percoll density gradient separation of subcellular components (including 'pure' mitochondria) of whole tissues (rat liver) and cell cultures (HepG2 cells). Lower volumes and protein concentrations were generally achieved following fractionation of cell-lines (as opposed to tissue), typically yielding poorer quality results. The purity of each subcellular compartment was confirmed following subcellular fractionation and percoll density gradient separation utilising antibodies to various known cellular components. Antibodies from various well-known manufacturers were utilised to allow comparison of staining accuracy during assessment of RAS components (for further information see Appendix Tables APP2. a. and APP2. b., pages 216-220). Each experiment was performed at least three times to ensure consistency of results.

R1.2.b. Imunofluorescence & Confocal Microscopy

HepG2 cells underwent processing and fixation, prior to antibody staining for immunofluorescence, utilising techniques already described (for further information see 'M5. Immunofluorescence'; page 76). Whole cells were incubated with primary antibodies to RAS components and known mitochondrial proteins, prior to incubating with fluorescent secondary antibodies and imaging on a confocal microscope.

Mitochondria were labelled using primary antibodies to ATP5b (the beta subunit of the F1 catalytic core located within the mitochondrial matrix), and Alexa Fluor (fluorescent green) secondary antibodies. Nuclei were labelled with Hoechst 33258, which emits a blue fluorescent light once excited by ultraviolet light. RAS components were similarly stained with secondary antibodies labelled with Alexa Fluor (for further information see Appendix Tables APP2. a. and APP2. b., pages 216-220).

R1.2.c. Immunogold Labelling & Electron Microscopy

Rat liver samples were processed utilising the LR white protocol and immunogold labelled by incubation with primary antibodies to RAS components and their requisite secondary antibodies (for further information see Appendix Tables APP2. a. and APP2. b., pages 216-220) before imaging via electron microscopy. High-quality electron microscopy was performed at the Electron Microscopy unit of the Royal Free Hospital (UCL Medical School, London), utilising freshly dissected rat liver tissue or trypsinised HepG2 cells.

Santa Cruz Biotechnology (Dallas, Texas, USA) primary antibodies were again utilised for ease of comparison with other experimental techniques and were used at manufacturer's recommended concentrations (see Appendix Tables APP2. a. and APP2. b., pages 216-220). Each experiment was performed at least three times to ensure consistency of results.

R1.3 Results

R1.3. a. Western Blotting

R1.3. a.i. Controls: Confirmation of purity of sub-cellular isolates

Mitochondrial isolation was performed utilising standardised and recognised techniques (for further information see 'M3. Cellular Sub-Fractionation & Mitochondrial Isolation'; page 68). Following each individual sub-fractionation, success of the process was confirmed using Western blotting and clear demonstration of well-established mitochondrial markers such as VDAC (voltage-dependent anion channel), a 280 amino acid porin ion channel located on the outer mitochondrial membrane (Shoshan-Barmatz et al., 2010); grp75 (glucose-regulated protein 75; mortalin), a member of the hsp70 family of chaperone/stress proteins located within the mitochondrial matrix (Lindquist, 1986); and COX-4, a crucial component of the last enzyme in the electron transport chain (cytochrome c oxidase, complex IV), located within the inner mitochondrial membrane (Bottinger et al., 2013).

Primary antibodies were sourced from a variety of different manufacturers and utilized at manufacturers' recommended concentrations (see Appendix Tables APP2. a. and APP2. b., pages 216-220). Antibodies to multiple cellular comppnents were utilized as a positive control to ensure the accuracy of subcellular fractionation and as the results below illustrated, mitochondrial markers were enriched in mitochondrial sub-fractions, demonstrating that the purity and accuracy of mitochondrial isolation was satisfactory (see Figure R1.1).

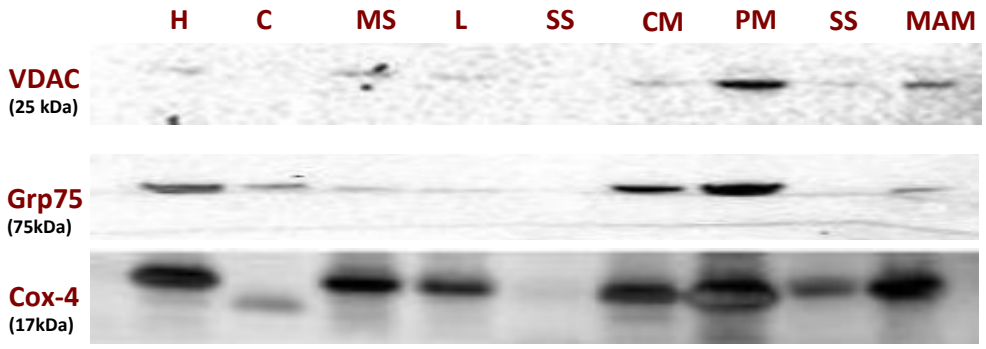

Figure R1.1. Representative Western blot of sub-fractionated rat liver demonstrates effective enrichment of known mitochondrial proteins in mitochondrial sub-fractions (H-Homogenate; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes; SS-Size Standards).

R1.3.a.ii. Investigation of RAS components in cellular sub-fractions

Once the composition of preparations had been confirmed utilising the above described technique, the various cellular fractions were stained for RAS components. Primary RAS antibodies were sourced from a variety of different manufacturers and utilized at manufacturers' recommended concentrations. Initially, antibodies from one manufacturer (Santa Cruz Biotechnology; Dallas, Texas, USA) were utilised to assess for the presence of RAS components in the various cellular compartments, including mitochondria. With this approach, Western blots revealed RAS components, including renin, AT_1R, AT_2R and angiotensin, within multiple sub-cellular compartments, including sub-fractionated mitochondrial compartments in both rat liver tissue and HepG2 cells (see Figures R1.2 & R1.3). However, ACE staining (both C-terminal and N-terminal antibodies) was absent from all mitochondrial sub-fractions in both rat liver tissue and HepG2 cells, at its normal molecular weight of 170-180kDa. Enrichment of a 55 kDa molecule, when stained with an ACE C-terminal antibody, was discovered within sub-fractionated mitochondrial compartments, potentially representing a novel, truncated, mitochondrial-specific form of ACE; possibly similar to the truncated mitochondrial form of renin described by

Clausmeyer *et al* (1999 and 2000). All findings were confirmed in at least three independent preparations.

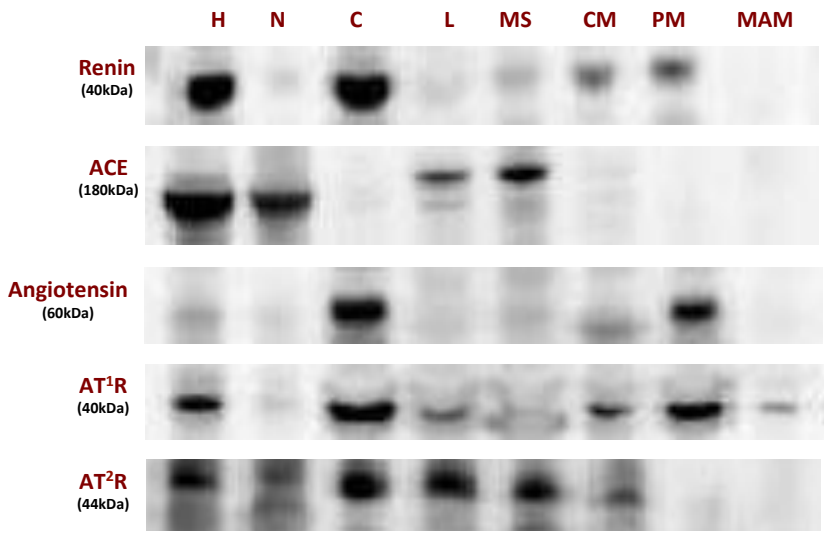

Figure R1.2. Representative Western blot of sub-fractionated rat liver tissue demonstrates staining for renin, angiotensin and AT_1R (rabbit polyclonal antibody) in a pure mitochondrial sub-fraction, renin, AT_1R and AT_2R staining in a crude mitochondrial fraction and AT_1R in a mitochondrial-associated membranes sub-fraction when Santa Cruz Biotechnology (Dallas, Texas, USA) antibodies are utilised. No consistent staining was seen for ACE (C-terminal) in either crude or pure mitochondrial fractions at its usual size of 180 kDa (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

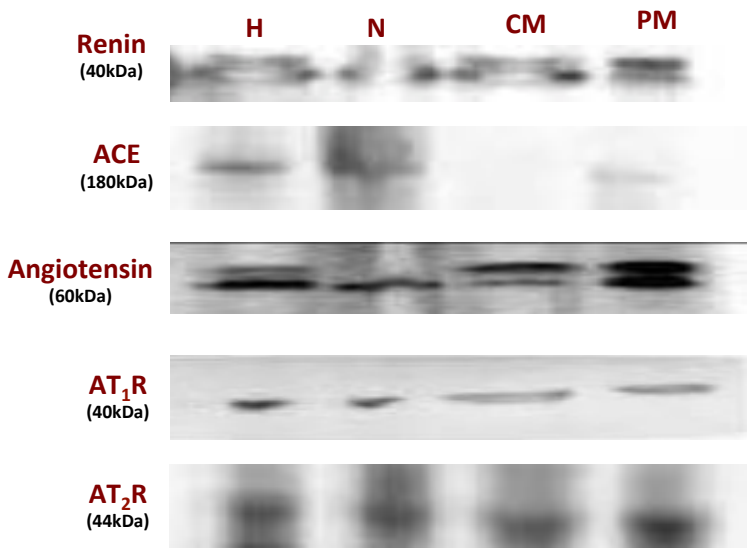

Figure R1.3. Representative Western blot of sub-fractionated HepG2 cells demonstrates renin, angiotensin, AT_1R (rabbit polyclonal) and AT_2R staining in crude and pure mitochondrial sub-fractions, when Santa Cruz Biotechnology (Dallas, Texas, USA) antibodies are utilised. No consistent staining was seen for ACE (C-terminal) in either crude or pure mitochondrial fractions (H-Homogenate; N-Nucleus; CM-Crude Mitochondria; PM-Pure Mitochondria).

R1.3. a.iii. Controls: Comparison of antibodies from different manufacturers

To assess the validity of an approach that utilises only one antibody, two antibodies were employed on a single, whole PVDF membrane (to assess non-specific bands, rather than just focussing on the molecular weight of interest) to compare results for each RAS component. As demonstrated below, inconsistent results were found following comparison (see Figures R1.4-R1.12).

Renin Western blots yielded very different results between the two antibodies utilised, with one preparation suggesting renin within a pure mitochondrial fraction, and one demonstrating a lack of mitochondrial renin. Similarly, AT_1R blots demonstrated contrasting results when two preparations from the same manufacturer were utilised, with

one antibody demonstrating AT_1R staining in CM, PM and MAM fractions, and another showing weak CM staining and no staining in PM or MAM fractions. However, AT_2R antibody use demonstrated the most consistent results, with both preparations demonstrating a CM band but no staining in PM or MAM fractions.

Ang antibodies from different manufacturers also demonstrated inconsistent results when applied to the same preparation; however, due to close molecular similarities between angiotensins and angiotensinogen, both antibodies are described by the manufacturers as binding to multiple angiotensin proteins including angiotensinogen, angiotensinogen precursor, ang I, ang II and ang III, potentially explaining the presence of multiple bands at various molecular weights.

As with the other RAS components, two initial ACE antibodies from the same manufacturer (one to the C-terminal and one to the N-terminal of the ACE molecule) were utilised to compare their similarity. Both antibodies demonstrated consistent staining at 170-195 kDa (usual ACE molecular weight) in homogenate and nuclear fractions. However, the C-terminal antibody also demonstrated an enriched 55 kDa band in crude and pure mitochondria, and mitochondrial-associated membrane sub-fractions. Two further ACE antibodies were therefore employed to further investigate this finding, with a C-terminal antibody from an alternate manufacturer delivering similar results with a 170 kDa molecule stained in the homogenate and nuclear fractions and a 55 kDa band in the CM fraction; whereas, an N-terminal antibody from an alternate manufacturer demonstrated multiple non-specific bands only.

Thus, most antibodies demonstrated multiple non-specific bands, casting doubt as to whether the bands representative of the sought molecular sizes actually represented the target molecule. Similarly, antibodies from different manufacturers, or of different preparations from the same manufacturer, tended to yield different results, and such lack of antibody specificity casts doubt on the validity of previously published findings and demonstrates the limitations of such molecular techniques.

Renin Preparations

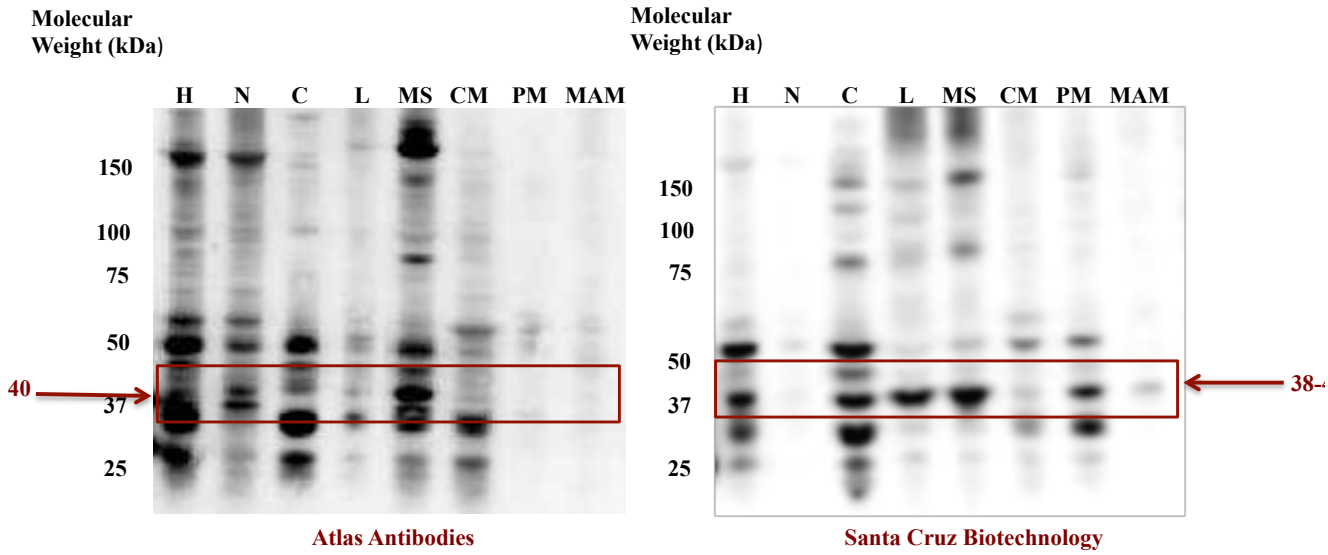

Figure R1.4. Representative whole Western blots of sub-fractionated rat liver tissue demonstrate inconsistent results when the same preparation was stained with renin antibody from different manufacturers (Santa Cruz Biotechnology (38-46 kDa) (Dallas, Texas, USA) and Atlas Antibodies (40 kDa) (Stockholm, Sweden)). The Santa Cruz antibodies suggest the presence of renin within a pure mitochondrial fraction, whereas the Atlas Antibodies preparation demonstrates a lack of renin staining within mitochondrial fractions and a large amount of non-specific bands at multiple molecular weights (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

ACE Preparations

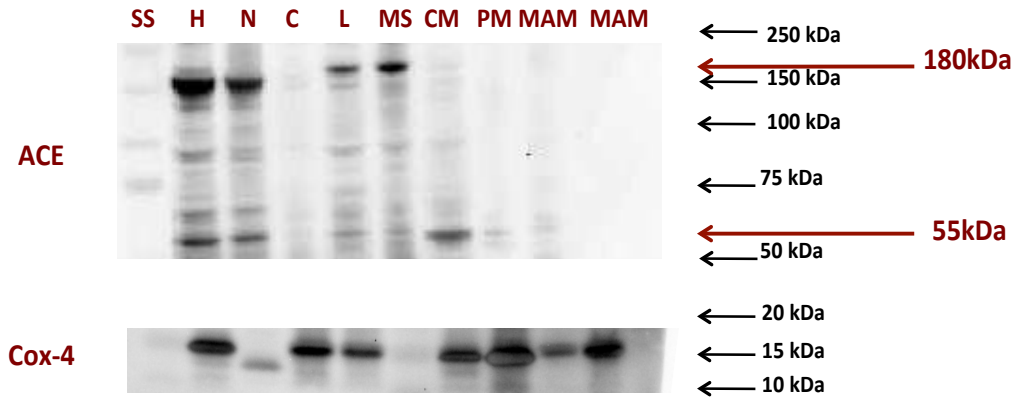

Figure R1.5. Representative Western blot of sub-fractionated rat liver tissue demonstrates consistent ACE staining at 180 kDa in the homogenate and nuclear fractions when an ACE (C-terminal) Santa Cruz Biotechnology (Dallas, Texas, USA) antibody was utilised. An enriched 55 kDa band in crude and pure mitochondria, and mitochondrial-associated membrane sub-fractions is also identified (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

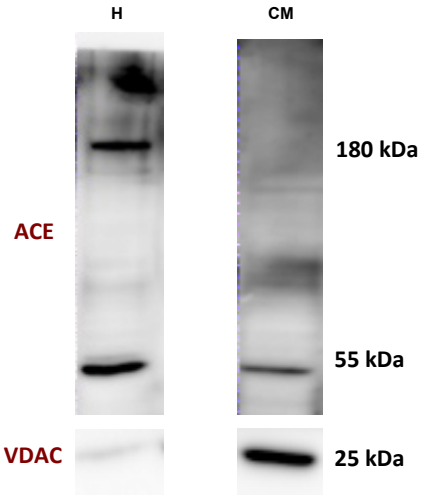

Figure R1.6. Repeat Western blot, utilising the same preparations and technique, but an enriched sub-fraction of rat liver tissue, again demonstrates ACE staining at both 180 kDa and 55 kDa in a crude mitochondrial sub-fraction when an ACE (C-terminal) Santa Cruz Biotechnology Dallas, Texas, USA) antibody was employed (H-Homogenate; CM-Crude Mitochondria).

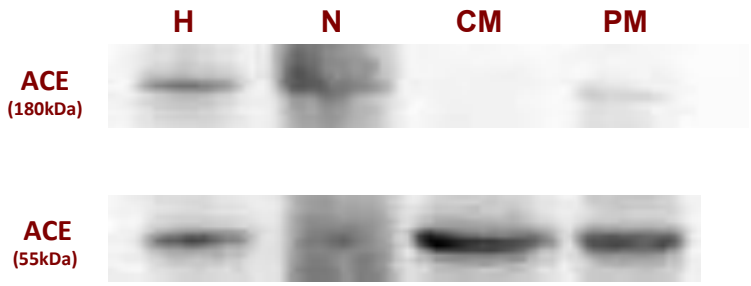

Figure R1.7. Representative Western blot of sub-cellular fractions from HepG2 cells demonstrates ACE staining in the homogenate at 180 kDa and at 55 kDa in both crude and pure mitochondrial sub-fractions when an ACE (C-terminal) Santa Cruz Biotechnology (Dallas, Texas, USA) antibody was used (H-Homogenate; N-Nucleus; CM-Crude Mitochondria; PM-Pure Mitochondria).

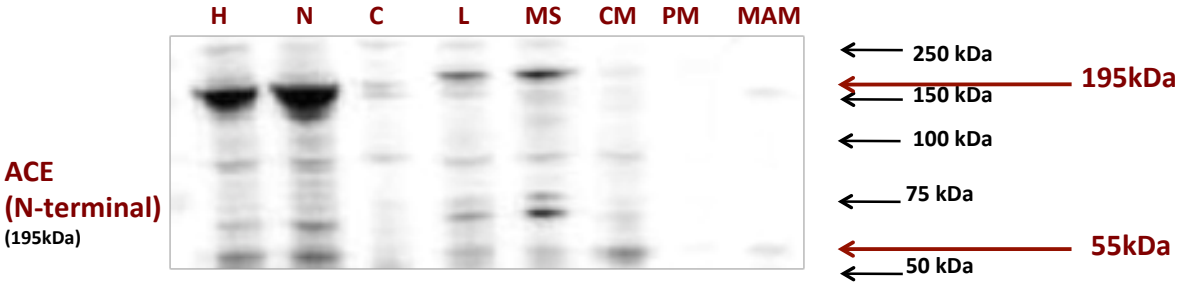

Figure R1.8. Representative whole Western blot of sub-cellular fractions of rat liver tissue demonstrates consistent ACE staining at 195 kDa in the homogenate and nuclear fractions, but a less convincing 55 kDa band in mitochondrial sub-fractions when an ACE (N-terminal) Santa Cruz Biotechnology (Dallas, Texas, USA) antibody was utilised (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

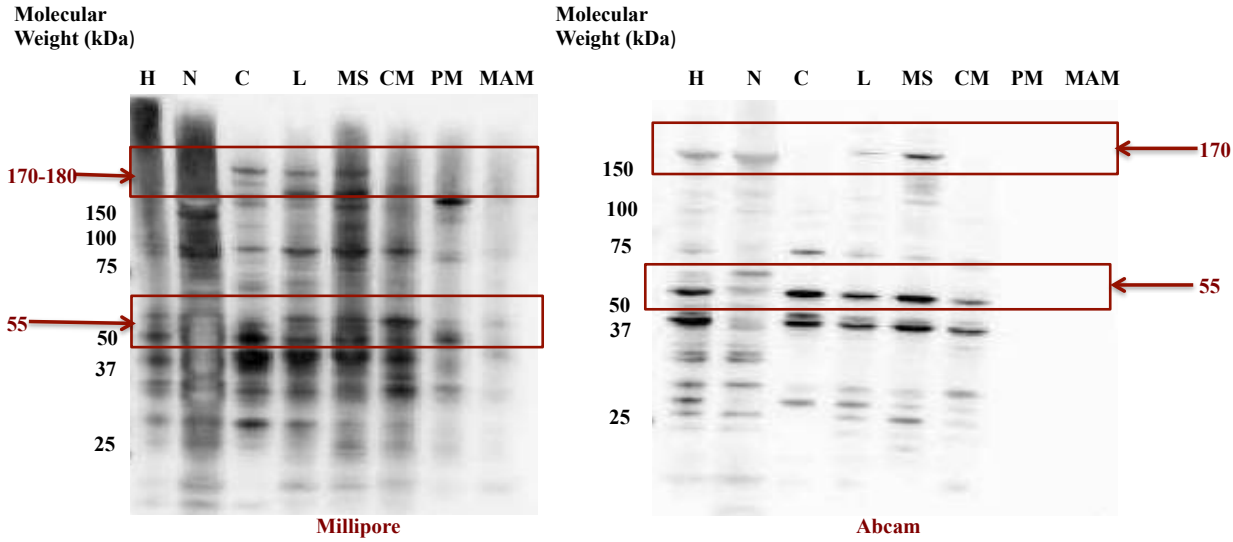

Figure R1.9. Representative whole Western blots of sub-fractionated rat liver tissue demonstrate inconsistent results when the same preparation was stained with ACE antibody from different manufacturers (Millipore (170-180 kDa) (Billerica, MA, USA) and Abcam (170 kDa) (Cambridge, UK)). The C-terminal Abcam antibody delivers similar results to the Santa Cruz C-terminal antibody, with staining for 170 kDa ACE in the homogenate and nuclear sub-fraction and 55 kDa staining in a crude mitochondrial fraction. The Millipore 9B9 clone antibody is directed to the N-terminal and provides a result with multiple non-specific bands at various molecular weights (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

Angiotensin Preparations

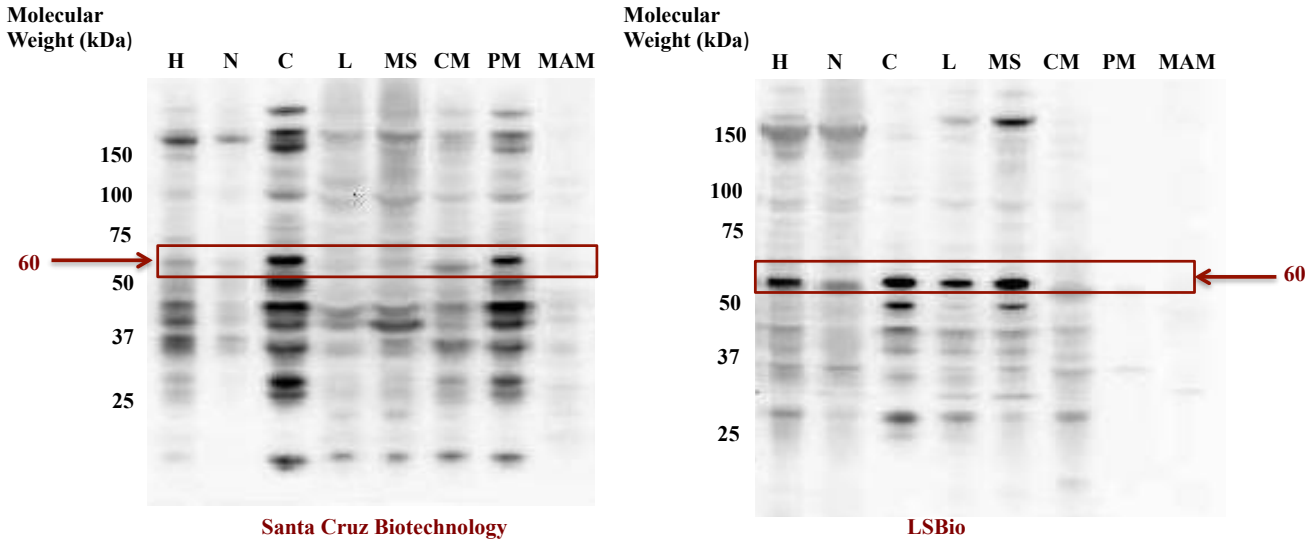

Figure R1.10. Representative whole Western blots of sub-fractionated rat liver tissue demonstrate inconsistent results when the same preparation was stained with angiotensin antibody from different manufacturers (Santa Cruz Biotechnology (Dallas, Texas, USA) and LS Bio (Seattle, WA, USA)). Due to the nature of the close molecular structure of angiotensins and angiotensinogen, both antibodies are described by the manufacturers as binding to multiple angiotensin proteins including angiotensinogen, angiotensinogen precursor, ang I, ang II and ang III; it may therefore be in keeping that multiple bands at various molecular weights are identified. The Santa Cruz antibody datasheet (http://datasheets.scbt.com/sc-7419.pdf)) describes an angiotensin of 60 kDa molecular weight stained with their antibody; whereas, the LSBio data sheet is less specific, describing instead that multiple molecular weights may be identified with the use of their antibody (http://www.lsbio.com/Documents/PDF/Antibodies/51294) (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

AT_1R Preparations

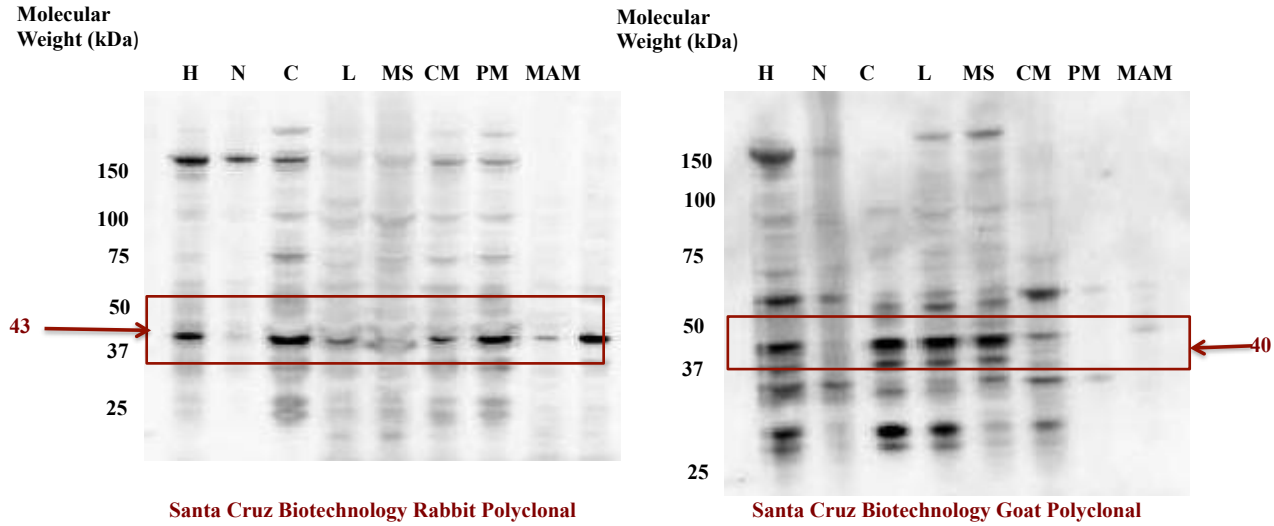

Figure R1.11. Representative whole Western blots of sub-fractionated rat liver tissue demonstrate inconsistent results when the same preparation was stained with different AT_1R antibody preparations from the same manufacturer (Santa Cruz Biotechnology, Dallas, Texas, USA). The rabbit polyclonal antibody (43 kDa) demonstrates AT_1R staining in CM, PM and MAM fractions; whereas, the goat polyclonal AT_1R antibody (40 kDa) demonstrates weak CM staining and no staining in PM or MAM fractions. Minimal other bands are identified, particularly with the rabbit polyclonal antibody (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

AT_2R Preparations

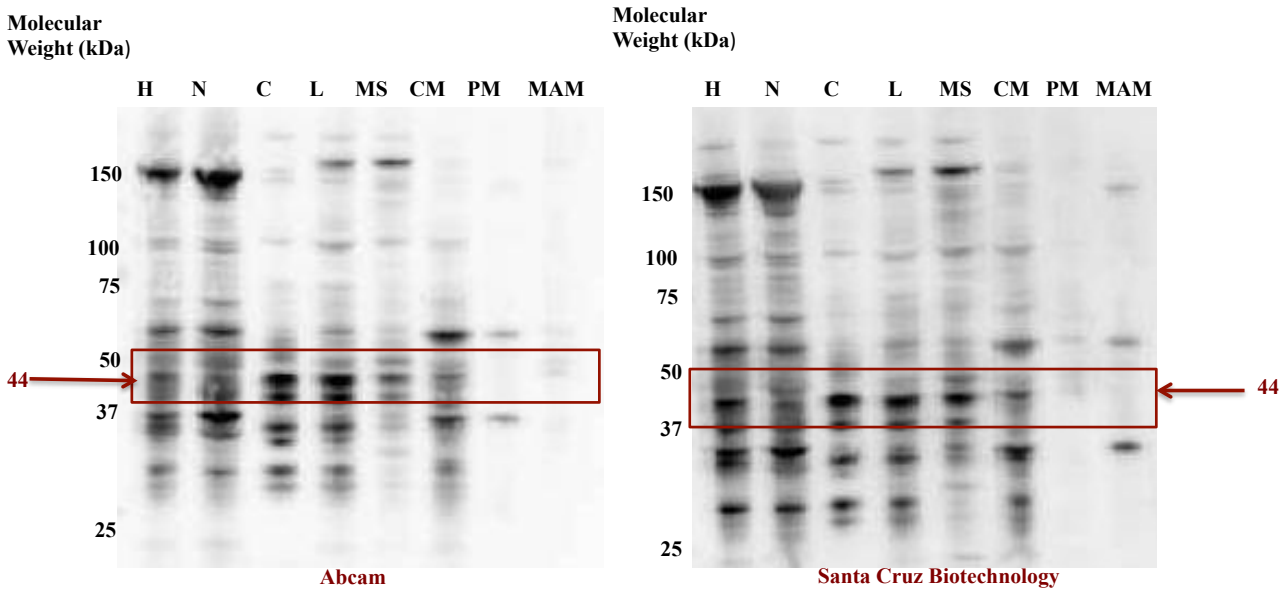

Figure R1.12. Representative whole Western blots of sub-fractionated rat liver tissue demonstrate consistent results when the same preparation was stained with AT_2R antibody from different manufacturers (Abcam (44 kDa) (Cambridge, UK) and Santa Cruz Biotechnology (44 kDa) (Dallas, Texas, USA)). Both antibodies demonstrate a weak CM band but no staining in PM or MAM fractions (H-Homogenate; N-Nucleus; C-Cytosol; MS-Microsomes; L–Lysosomes; CM-Crude Mitochondria; PM-Pure Mitochondria; MAM-Mitochondrial Associated Membranes).

R1.3. b. Immunofluorecence & Confocal Microscopy

R1.3. b.i. Controls: Immunofluorescence of known cellular proteins

Clear, specific immunofluorescence of both mitochondria and nuclei was observed utilising antibodies to ATP5b and Hoechst 33258 respectively, demonstrating that cellular fixation and processing was sufficient to allow immunofluorescence without destroying intracellular proteins (see Figure R1.13).

Santa Cruz Biotechnology primary RAS antibodies were utilised to provide consistency with Western blot results. Primary and secondary antibodies were utilized at manufacturers recommended concentrations (see Appendix Tables APP2.a. and APP2.b., pages 216-220).

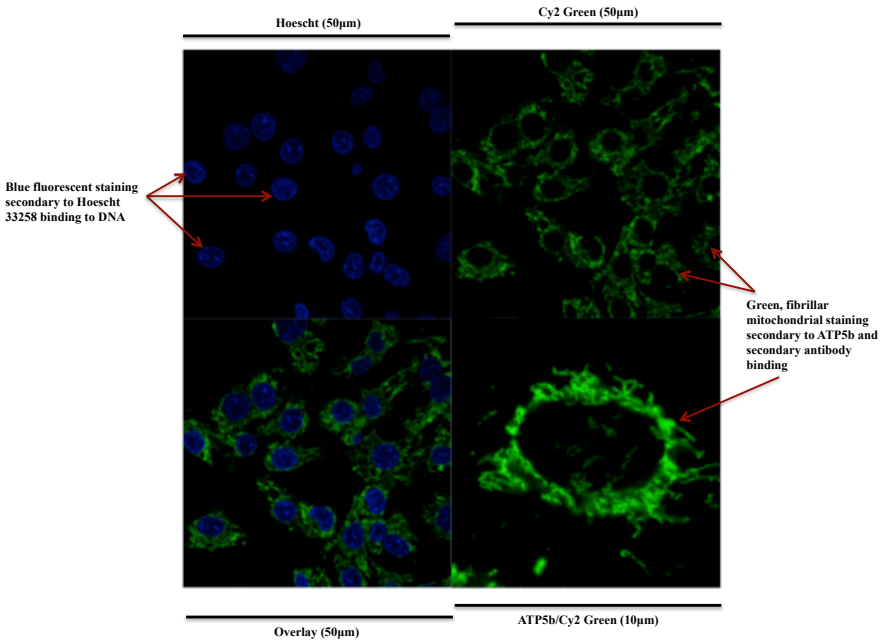

Figure R1.13. The use of Hoechst 33258 antibody (blue immunofluorescence) and ATP5b primary antibody with Cy2 green secondary antibody demonstrates clear blue nuclear immunofluorescence and clear green mitochondrial immunofluorescence within HepG2 cells, thus demonstrating the maintenance of structural integrity of mitochondrial proteins, with this processing and fixation technique.

R1.3. b.ii. Immunofluorescence of intra-cellular RAS components

Immunofluorescence of RAS components was performed in conjunction with ATP5b and Hoechst 33258 to observe for the presence of RAS components and any co-localisation with mitochondrial (ATP5b) or nuclear (DNA) contents. A fluorescent signal following labelling was present for all classical RAS components (e.g. renin, ACE, ang, AT_1R and AT_2R) within HepG2 cells (see Figures R1.14-R1.18). Further, renin, ang and ACE appeared to co-localise with mitochondria, although there was no obvious co-localisation within mitochondria when antibodies to AT_1R and AT_2R were utilised. The presence of minimal non-specific immunofluorescence was confirmed by performing negative controls with primary antibody and secondary antibody alone (see Figures R1.19-R1.20).

However, the staining identified with these antibodies and this technique could be described as relatively non-specific, having no particular pattern of staining. This may suggest that significant non-specific primary antibody binding is present, a hypothesis that concurs with the fact that multiple non-specific bands were seen when these antibodies were utilised for Western blotting of sub-fractionated rat liver tissue. Alternatively, these findings may simply reflect the fact that many of the RAS components under investigation are ubiquitous throughout multiple cellular compartments.

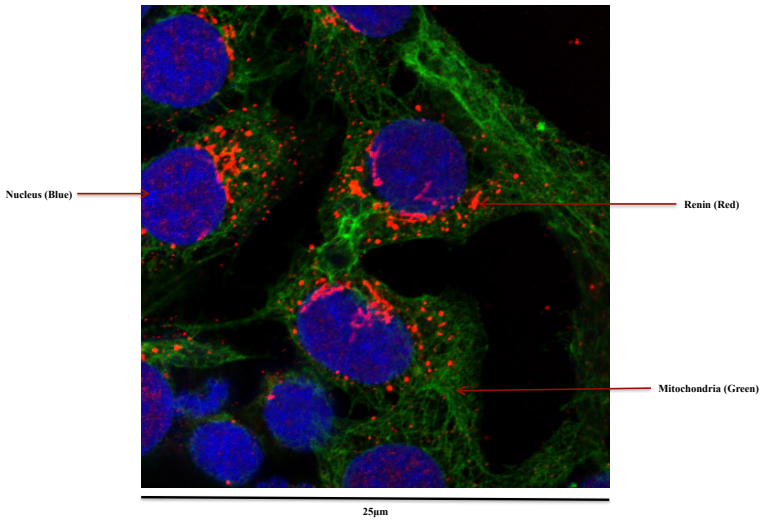

Figure R1.14. Image of representative staining with renin primary antibody and AlexaFluor® secondary antibody (red immunofluorescence), in conjunction with ATP5b (green immunofluorescence) and Hoechst 33258 (blue immunofluorescence) demonstrates renin within HepG2 cells, where it co-localises with both nuclei and mitochondria.

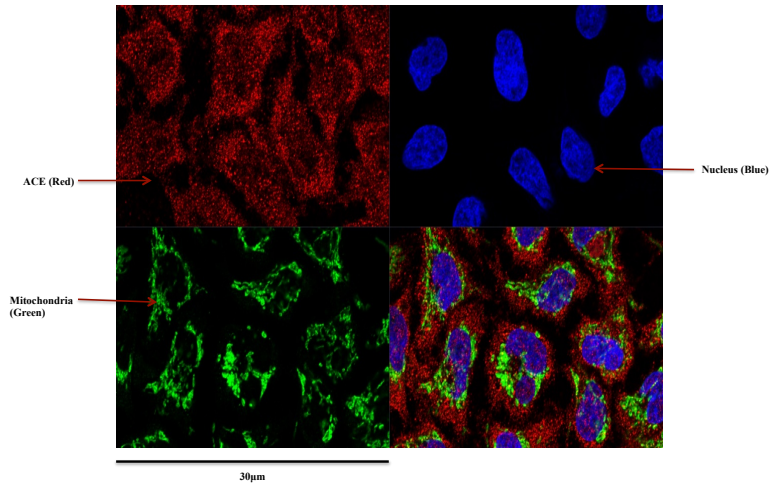

Figure R1.15. Image of representative staining with ACE primary antibody and AlexaFluor® secondary antibody (red immunofluorescence), in conjunction with ATP5b (green immunofluorescence) and Hoechst 33258 (blue immunofluorescence) staining, demonstrates ubiquitous ACE immunofluorescence within nuclei and some co-localisation with mitochondria. ACE is also demonstrated at the periphery of the cell.

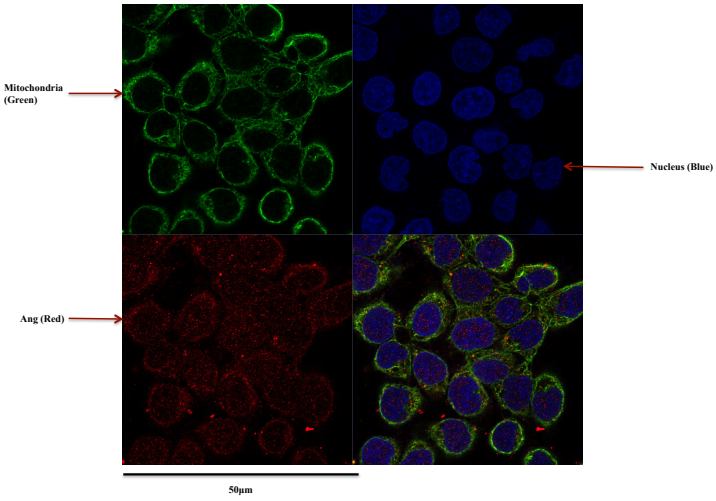

Figure R1.16. Image of representative staining with angiotensin primary antibody and AlexaFluor® secondary antibody (red immunofluorescence), in conjunction with ATP5b (green immunofluorescence) and Hoechst 33258 (blue immunofluorescence) staining, demonstrates ubiquitous angiotensin immunofluorescence within HepG2 cells, with co-localisation in both nuclei and mitochondria.

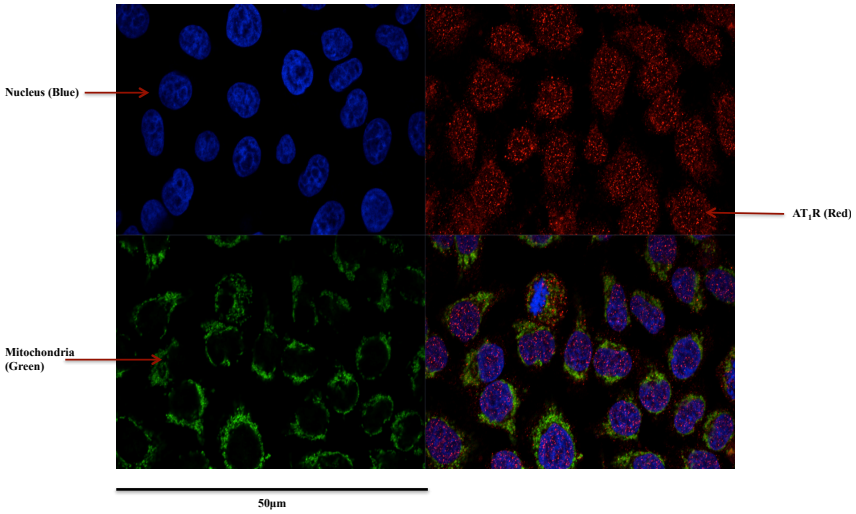

Figure R1.17. Image of representative staining with AT_1R primary antibody and AlexaFluor® secondary antibody (red immunofluorescence), in conjunction with ATP5b (green immunofluorescence) and Hoechst 33258 (blue immunofluorescence) staining, demonstrates AT_1R immunofluorescence largely within the nuclei of HepG2 cells, with minimal co-localisation within the mitochondria.

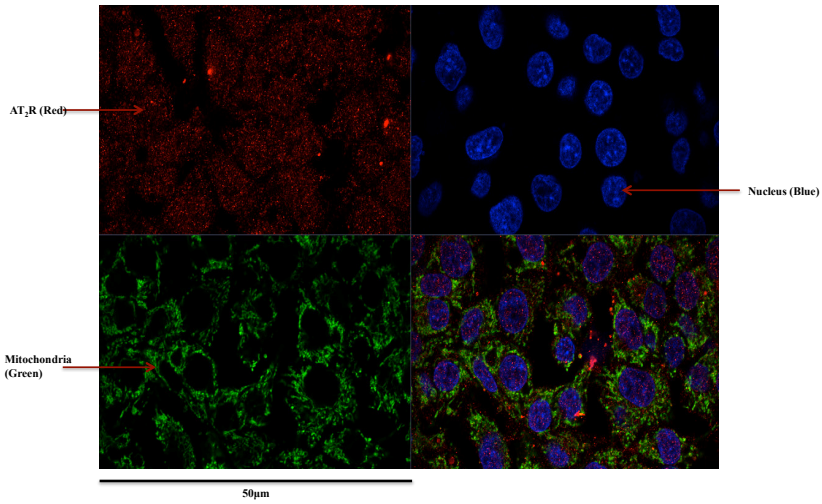

Figure R1.18. Image of representative staining with AT_2R primary antibody and AlexaFluor® secondary antibody (red immunofluorescence), in conjunction with ATP5b (green immunofluorescence) and Hoechst 33258 (blue immunofluorescence) staining, again demonstrates AT_2R immunofluorescence within the nuclei of HepG2 cells, with minimal co-localisation with mitochondria.

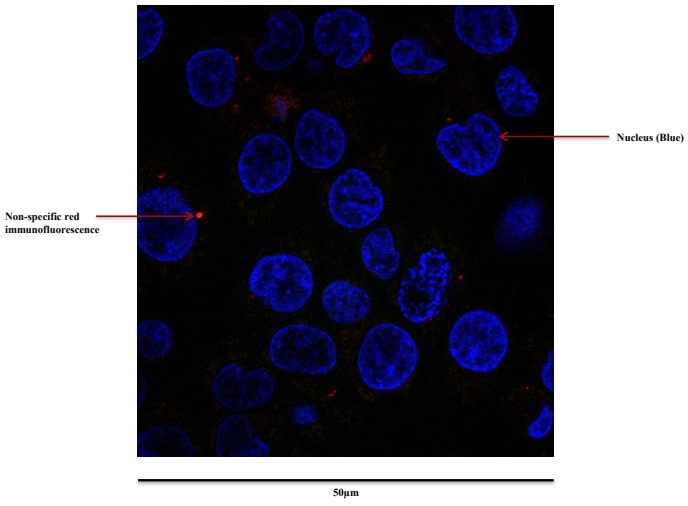

Figure R1.19. Representative negative control image utilising ACE primary antibody alone and Hoechst 33258 (blue immunofluorescence) demonstrates minimal non-specific red immunofluorescence. Similar results were demonstrated when other RAS component primary antibodies were utilised.

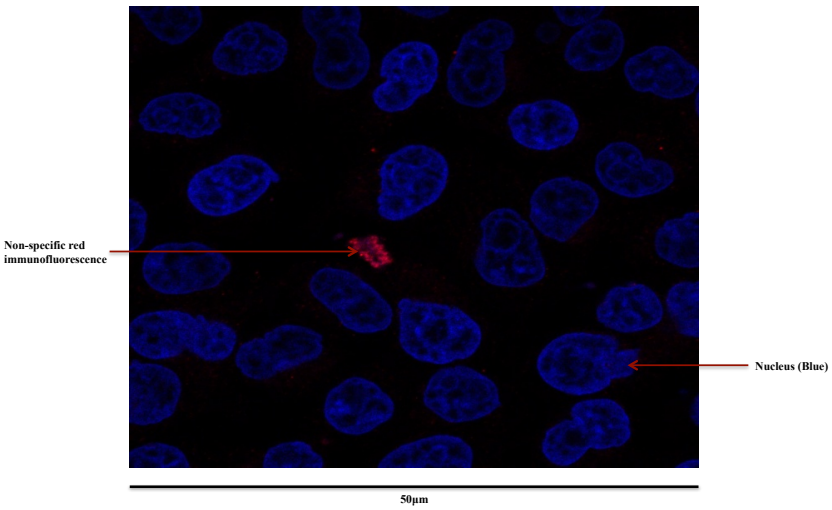

Figure R1.20. Representative negative control image utilising AlexaFluor® secondary antibody alone and Hoechst 33258 (blue immunofluorescence) demonstrates minimal non-specific secondary antibody binding and red immunofluorescence. Similar results were demonstrated when other secondary antibodies were utilised.

R1.3. c. Immunogold Labelling & Electron Microscopy

R1.3. c.i. Electron microscopy of RAS components in HepG2 cells

Using immunogold labeling and electron microscop1, ACE was shown to be associated with the mitochondria of HepG2 cells (see Figures R1.21-R1.27). Staining also visible elsewhere within the cell might be non-specific, or might simply reflect a ubiquitous distribution for ACE.

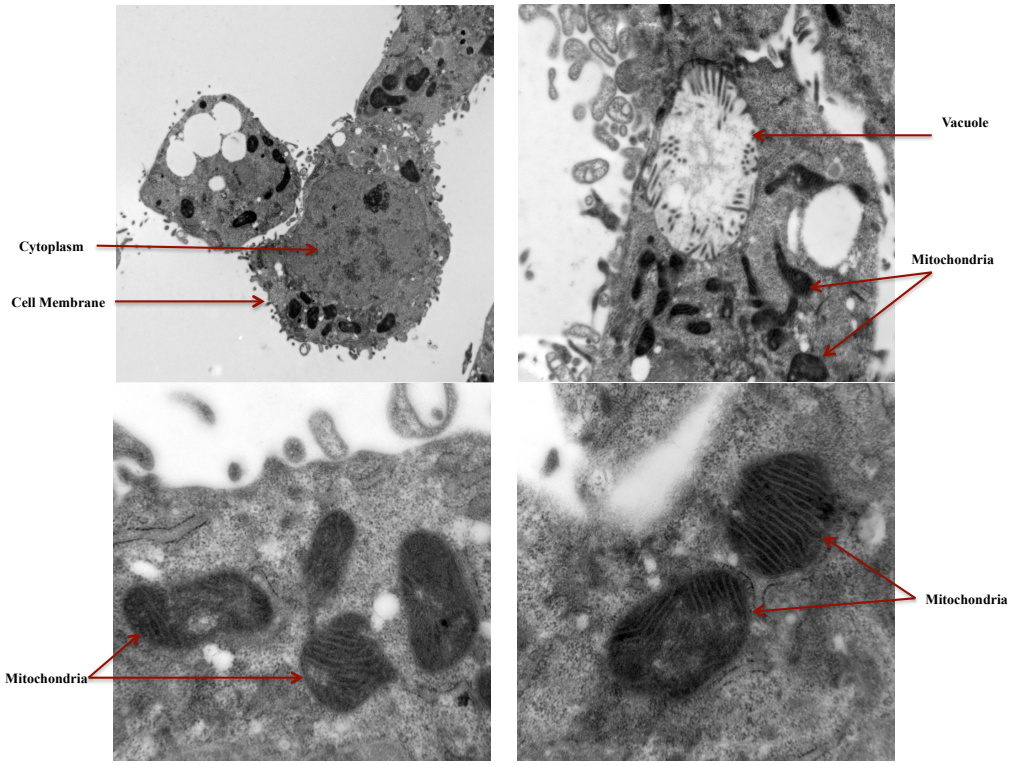

Figure R1.21. Representative control images of HepG2 cells with primary or secondary antibody only, demonstrate little or no non-specific staining following electron microscopy (5600-40,000 x magnification). Upper Left Image: overall view of hepatocyte; Upper Right Image: close up of hepatocyte, demonstrating large vacuole and multiple mitochondria; Bottom Images: closer inspection of mitochondria, demonstrating multiple cristae.

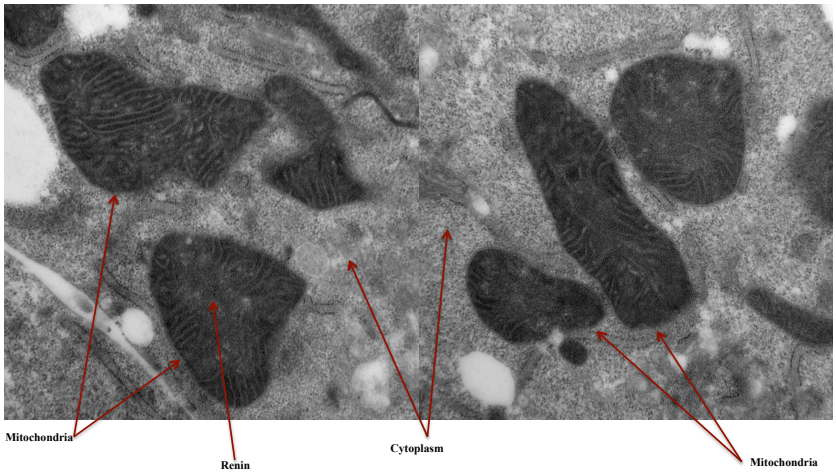

Figure R1.22. Renin immunogold labelling of HepG2 cells with renin antibody and appropriate secondary antibody demonstrates minimal renin presence (black dots) in association with mitochondria (both images 31,000 x magnification). In fact, only one immunogold particle is identified within mitochondria and very little staining is identified throughout the cell.

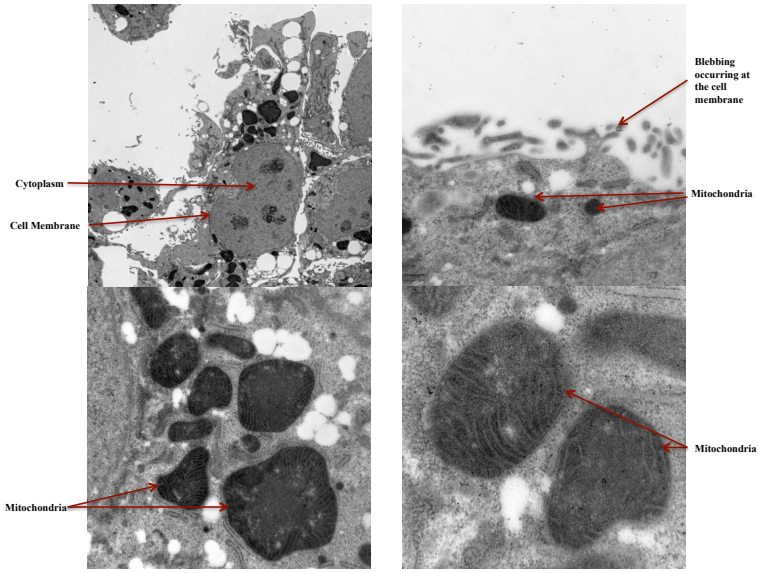

Figure R1.23. ACE immunogold labelling of HepG2 cells with ACE (C-terminal) antibody and appropriate secondary antibody demonstrates the presence of ACE (black dots) in association with mitochondria (3400-53,000 x magnification). Further staining can also be identified throughout the cell, as might be expected from a ubiquitous molecule. Upper Left Image: overall view of hepatocytes; Upper Right Image: close up of cell membrane; Bottom Images: closer inspection of mitochondria.

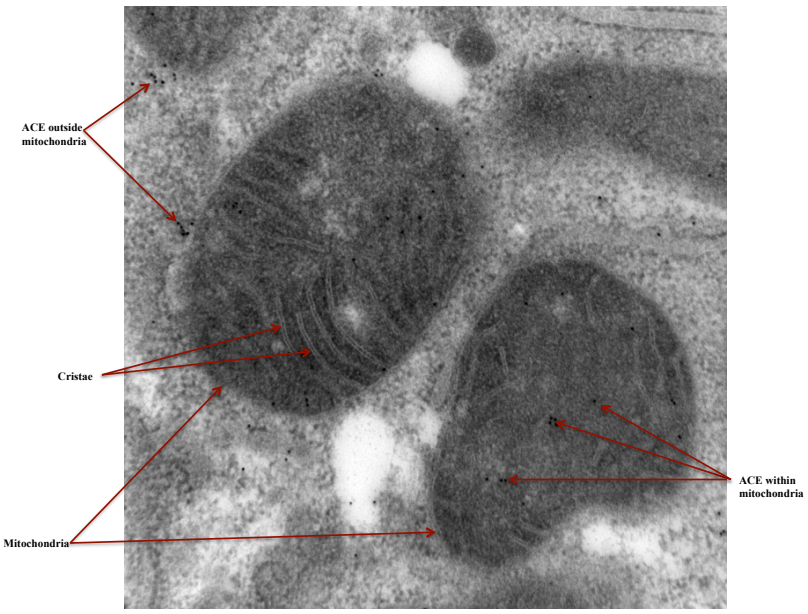

Figure R1.24. ACE immunogold labelling of HepG2 cells with ACE (C-terminal) antibody and appropriate secondary antibody demonstrates the presence of ACE (black dots) in association with mitochondria (53,000 x magnification). Further staining can be identified throughout the cell, as might be expected from a ubiquitous molecule.

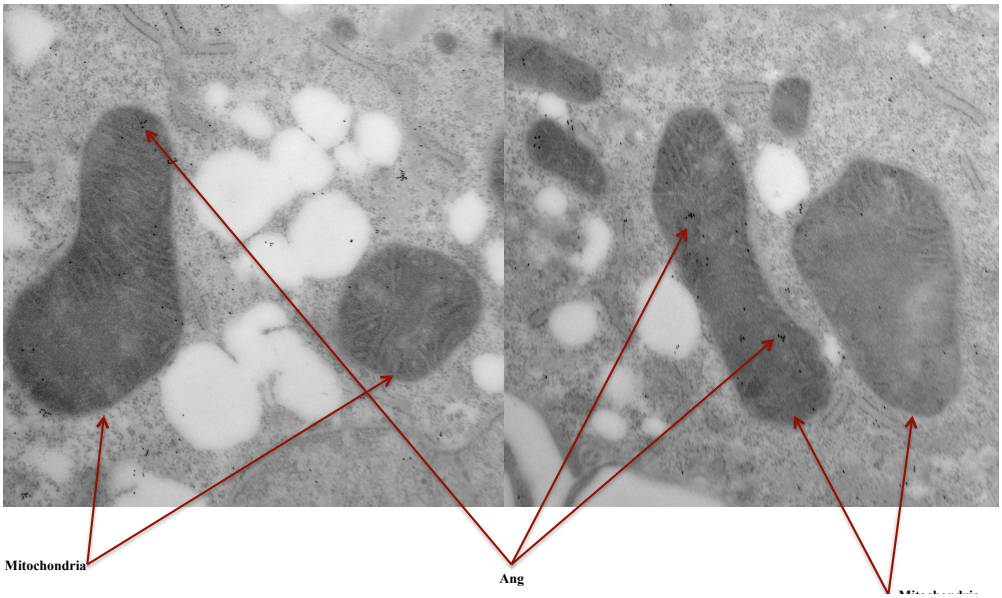

Figure R1.25. Ang immunogold labelling of HepG2 cells with ang antibody and appropriate secondary antibody demonstrates the presence of ang (black dots) in association with mitochondria (31,000 x magnification). Further staining can be identified throughout the cell, as might be expected from a ubiquitous molecule.

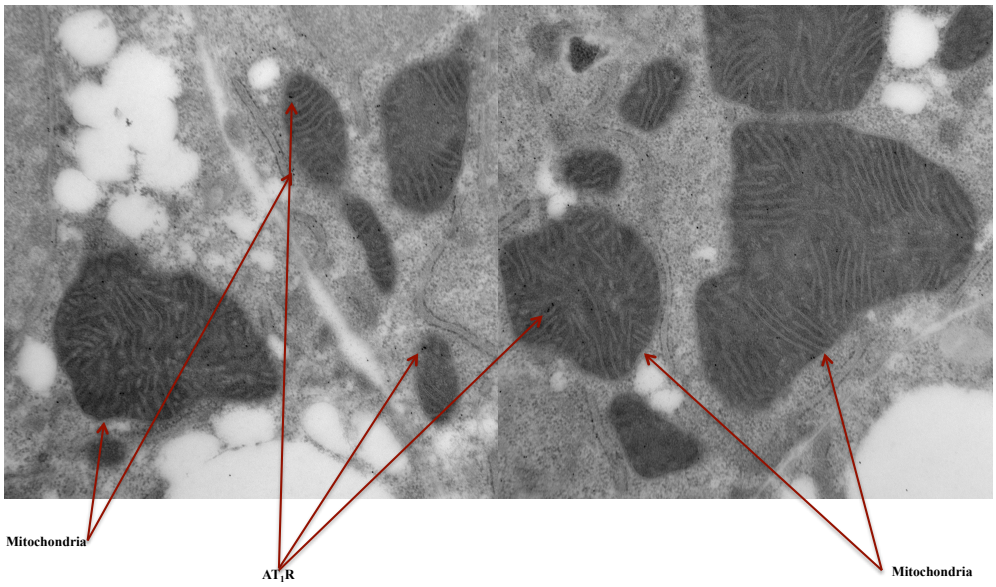

Figure R1.26. AT_1R immunogold labelling of HepG2 cells with AT_1R antibody and appropriate secondary antibody demonstrates the presence of AT_1R (black dots) in association with mitochondria (31,000 x magnification). Further staining can be identified throughout the cell, as might be expected from a ubiquitous molecule.

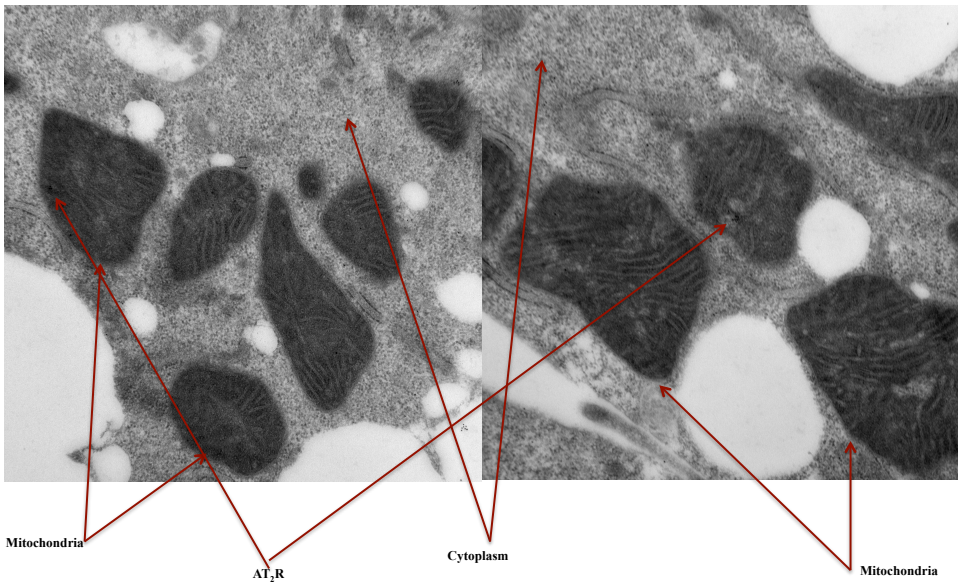

Figure R1.27. AT_2R immunogold labeling of HepG2 cells with AT_2R antibody and appropriate secondary antibody demonstrates the presence of AT_2R (black dots) in association with mitochondria (31,000 x magnification). Further staining can be identified throughout the cell, as might be expected from a ubiquitous molecule.

R1.3.c.ii. Electron microscopy of RAS components in rat liver tissue

Using immunogold labeling and electron microscopy, ACE was shown to be associated with the mitochondria of rat liver tissue (see Figures R1.28-R1.33). Staining also visible elsewhere within the cell might be non-specific, or might simply reflect a ubiquitous distribution of ACE.

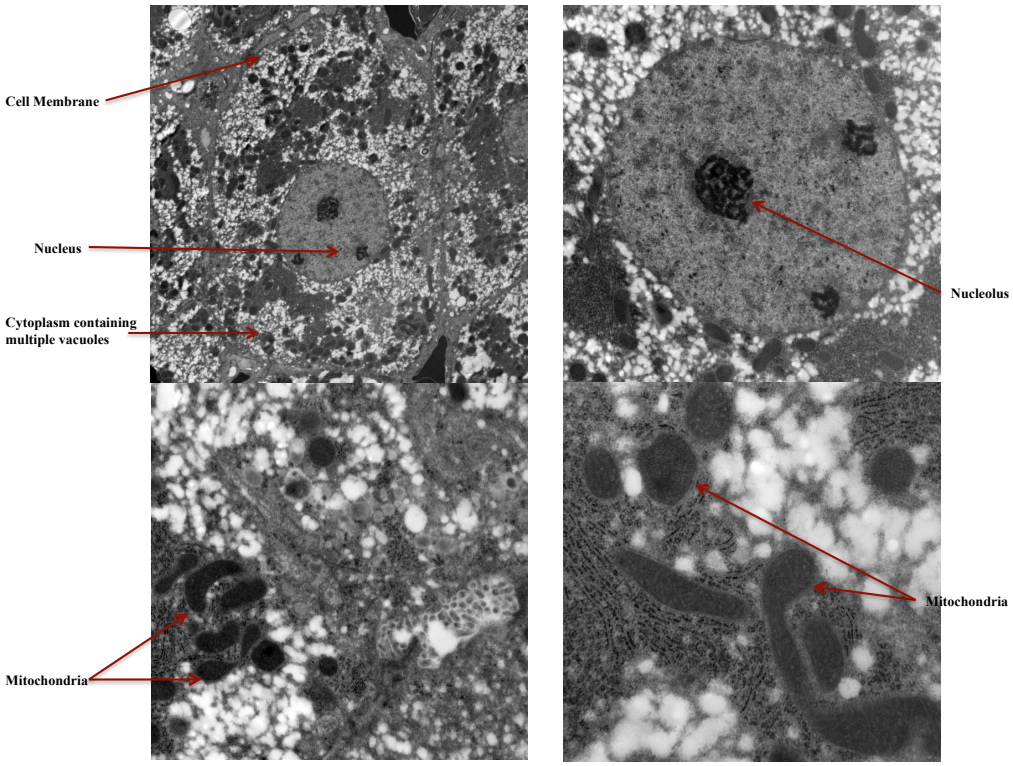

Figure R1.28. Representative control images of rat liver tissue sections with primary or secondary antibody only demonstrate little or no non-specific staining following electron microscopy (3400-31,000 x magnification). Upper Left Image: overall view of hepatocyte; Upper Right Image: close up of nucleus; Bottom Images: closer inspection of mitochondria.

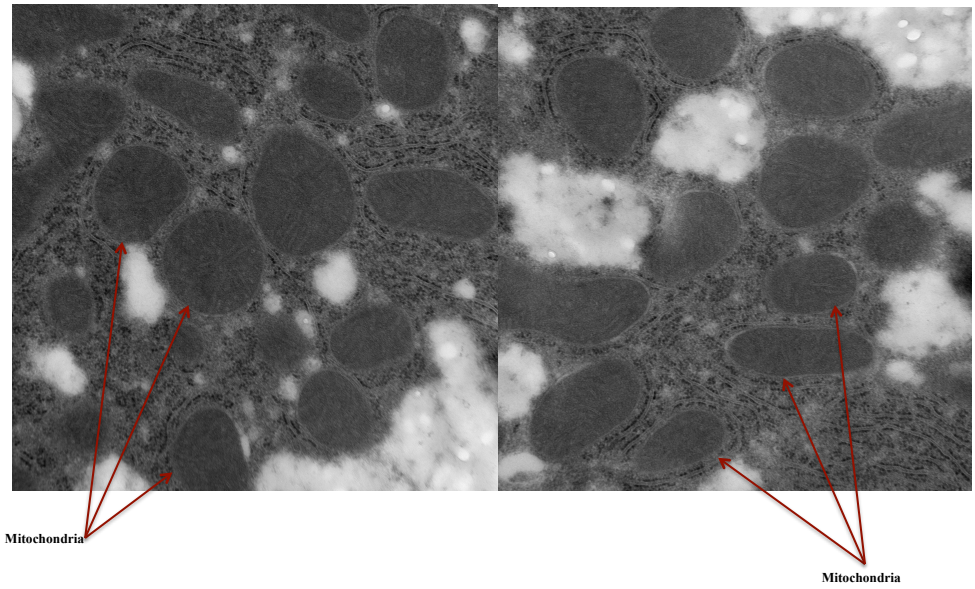

Figure R1.29. Renin immunogold labeling of rat liver tissue sections with renin antibody and appropriate secondary antibody demonstrates a lack of renin presence in association with mitochondria upon electron microscopy (31,000 x magnification).

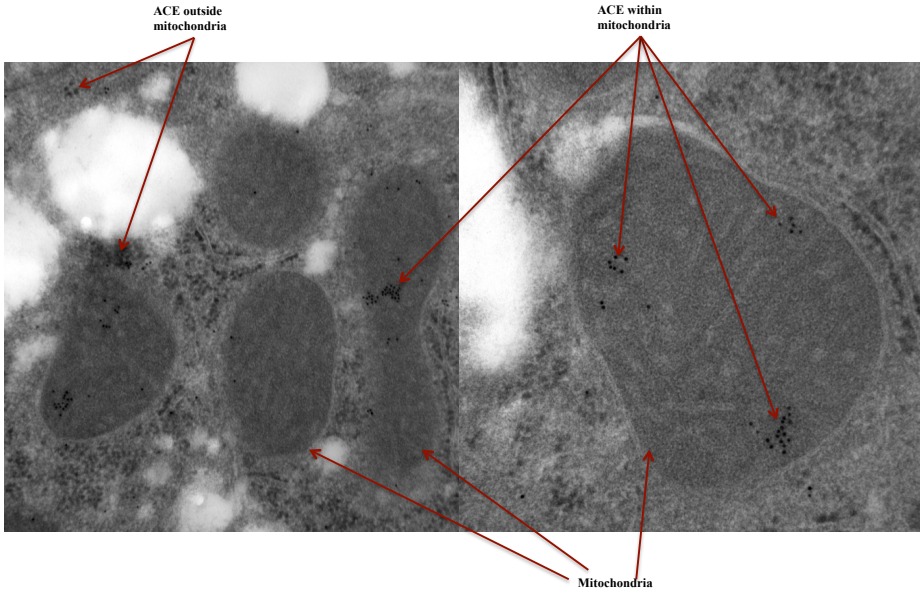

Figure R1.30. Immunogold labelling of rat liver tissue sections with ACE (C-terminal) antibody and appropriate secondary antibody demonstrates the presence of ACE (black dots) in association with mitochondria upon electron microscopy (31,000-88,000 x magnification). Further staining can be identified throughout the cell, as might be expected from a ubiquitous molecule.

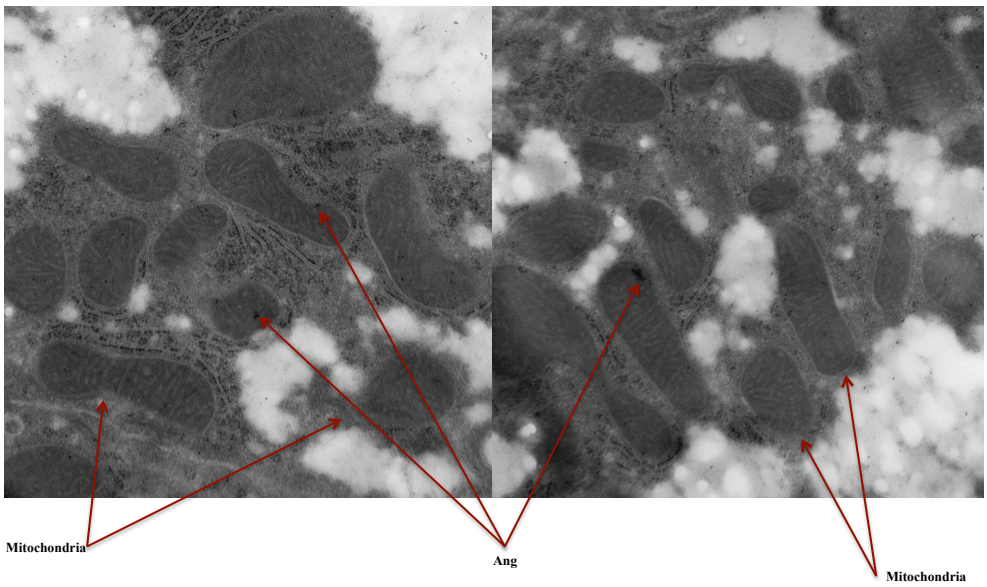

Figure R1.31. Immunogold labeling of rat liver tissue sections with ang antibody and appropriate secondary antibody demonstrates the presence of ang (seen here as black dots) in association with mitochondria upon electron microscopy (31,000 x magnification). However, further staining can also be identified throughout the cell, as might be expected from a ubiquitous molecule.

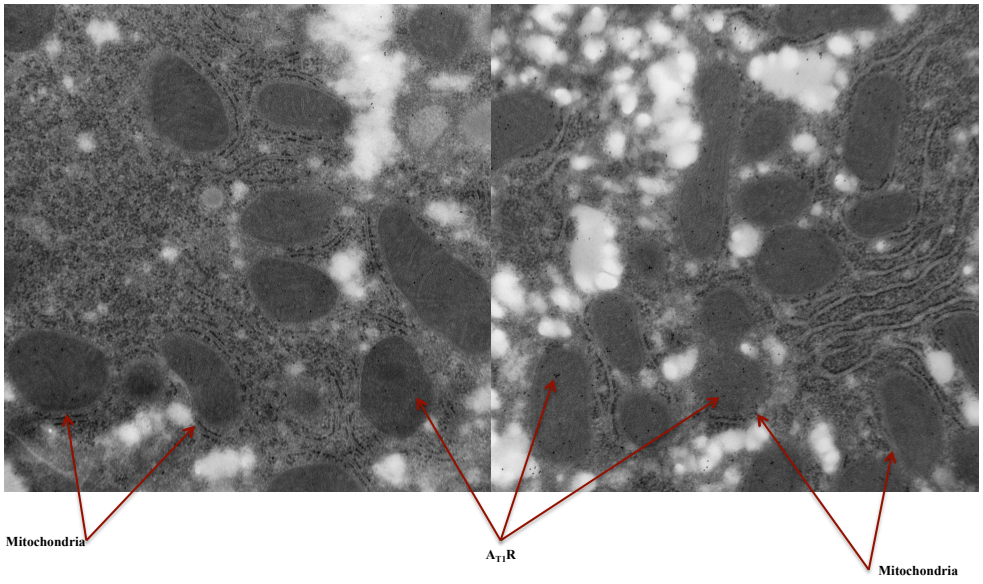

Figure R1.32. Immunogold labeling of rat liver tissue sections with AT_1R antibody and appropriate secondary antibody demonstrates the presence of AT_1R (seen here as black dots) in association with mitochondria upon electron microscopy (31,000 x magnification). Further staining can also be identified throughout the cell.

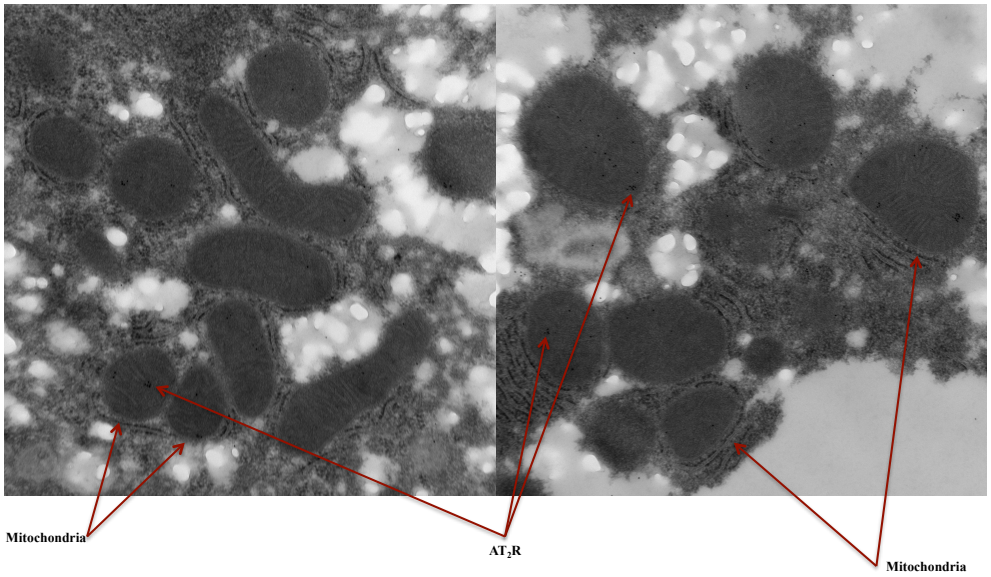

Figure R1.33. Immunogold labelling of rat liver tissue sections with AT_2R antibody and appropriate secondary antibody demonstrates the presence of AT_2R (seen here as black dots) in association with mitochondria upon electron microscopy (31,000 x magnification). Further staining can also be identified throughout the cell.

R1.3.d. Summary of Immmunohistochemical techniques for the mitochondrial localization of RAS

	WB	IF	EM
Renin	Y	Y	N
ACE	N	Y	Y
Ang	Y	Y	Y
AT_1R	Y	N	Y
AT_2R	Y	N	Y

Table R1.6. The presence of RAS components within hepatic mitochondria was inconsistent, utilising these techniques, with different strategies suggesting different results (WB- Western Blotting; IF- Immunofluorescence; EM- Electron Microscopy).

R1.4 Discussion

Taken together, the data presented in this chapter suggest the possibility of certain RAS components being present within mitochondria; however, the results are inconsistent between techniques and therefore cannot provide unequivocal evidence for a full mitochondrial RAS, due to the inherent limitations of the antibodies and methods. Western blotting demonstrated renin, AT_1R, AT_2R and angiotensin within sub-fractionated mitochondrial compartments in both rat liver tissue and HepG2 cells; whilst ACE, at its usual size of 170-180 kDa was absent from all mitochondrial sub-fractions in both rat liver tissue and HepG2 cells. However, further analysis demonstrated an enrichment of a 55 kDa molecule, when stained with ACE C-terminal antibodies, from two different manufacturers, within sub-fractionated crude mitochondrial compartments; potentially representing a novel, mitochondrial-specific form of ACE. Immunofluorescence and confocal microscopy of HepG2 cells demonstrated the presence of all classical RAS components (e.g. renin, ACE, ang, AT_1R and AT_2R) within HepG2 cells, with renin, ang and ACE co-localising with mitochondria. Immunogold labelling and electron microscopy demonstrated ACE, ang, AT_1R and AT_2R in association with rat liver mitochondria and mitochondria of the HepG2 cell line.

The immunohistochemical techniques utilised are reliant upon the accuracy of cell/tissue processing, to allow the structural integrity of the molecules under investigation (RAS components) to be maintained. They also rely upon the specificity of the antibodies employed, to eradicate non-specific background staining, thought to result from attraction of primary and secondary antibodies to endogenous Fc receptors (Buchwalow et al., 2011). The strategies followed here (and within the previous studies describing RAS components within mitochondria) incorporated a blocking step (goat serum or bovine serum albumin), thought to prevent the hydrophobic interactions between proteins and ionic or electrostatic interactions (Buchwalow et al., 2011). However, recent studies suggest that such widely-utilised protocols are based upon conflicting and outdated reports and may therefore be ineffective (Buchwalow et al., 2011). The antibodies employed here demonstrated inconsistent findings between Western blotting, immunofluorescence and electron microscopy. However, electron microscopy results between cell-lines and tissue were

consistent both with each other and the findings of other authors, such as Abadir *et al* (2011) and Peters *et al* (1996). In contrast with the consistency of EM was the presence of multiple bands when the same antibodies are utilised for Western blotting and relatively non-specific patterns of staining when utilised for immunofluorescence, both of which reinforce the idea that non-specific, background staining may be a particular limitation of this technique and these antibodies. Further, antibodies from different manufacturers yielded different results when utilised for Western blotting, calling into question the conclusions from past studies, which relied only upon one antibody. These results therefore cannot confirm the findings of Abadir *et al* (2011), who suggested that the AT_2R might be located upon the inner mitochondrial membrane. Similarly, without a demonstration of their exact site, hypotheses regarding the potential functional role of postulated intra-mitochondrial RAS proteins become redundant. Alternative techniques will therefore be necessary to provide irrefutable proof that RAS components are present within mitochondria, given such limitations of immunohistochemical techniques.

One consistent finding was the detection of a 55 kDa band when CM preparations were stained with an ACE C-terminal, but not an ACE N-terminal antibody; suggesting that if this band did represent a mitochondrial form of ACE, it may be proceesed or cleaved to remove the N-terminal. Previous studies have suggested that RAS components may require significant processing to enter the mitochondria and a novel, truncated form of non-secretory renin lacking 36 amino acids (including the complete ER signal) has been reported to be present within the mitochondria of the rat adrenal gland (Clausmeyer et al., 2000, Clausmeyer et al., 1999, Wanka et al., 2009). This 55 kDa molecule may potentially represent a novel, truncated form of ACE; however, this molecular weight is significantly below the usual size of ACE (170-195 kDa) and would necessitate significant cleavage and processing of the ACE molecule prior to mitochondrial import, potentially rendering its enzymatic activity non-functional and raising the question of what its function may subsequently be. Further investigation of the nature of this 55 kDa molecule was performed, via isolation and characterisation of the molecule of interest (see Results 2: Is there a mitochondrial-specific form of ACE).

RESULTS 2:

IS THERE A MITOCHONDRIAL-SPECIFIC FORM OF ACE?

Abstract

Introduction: Mitochondrial RAS proteins were inconsistently identified in initial experiments, results appearing dependent upon the investigative strategy used and the antibody employed. Whilst the normal molecular weight of ACE is 180-195 kDa, Western blots consistently identified a 55 kDa molecule which stained with an ACE (C-terminal) antibody in mitochondrial (particularly CM) fractions, potentially representing a novel, specific, truncated, intra-mitochondrial form of ACE. For further investigation the 55 kDa band was isolated, purified and concentrated to allow proteomic analysis by mass spectrometry.

Methods: Immunoprecipitation was performed on rat liver crude mitochondrial sub-fractions to isolate and concentrate the 55 kDa form of ACE. Deglycosylation of the band of interest was also performed to reduce heterogeneity of mass and charge in the sample and facilitate subsequent mass spectrometry via MALDI-TOF.

Results: Immunoprecipitation of rat liver tissue and ACE (C-terminal) antibody demonstrated consistent concentration of a 50 kDa band, whereas deglycosylation of this immunoprecipitation eluate demonstrated a subsequent consistent reduction in mass to 30 kDa. Database searches following mass spectrometry of 55 kDa, 50 kDa and 30 kDa bands demonstrated no results consistent with ACE protein.

Discussion: Smaller ACE isoforms than the usual size of 170-195 kDa have been previously described, as has a truncated intra-mitochondrial renin protein. Negative mass spectrometry results, as presented here, indicate that that the 55 kDa band is most likely to represent non-specific binding by the ACE (C-terminal) antibody and although these results do not completely exclude the presence of ACE in low abundance, they do render the presence of ACE in the mitochondria unlikely.

R2.1 Introduction

Mitochondrial RAS proteins were inconsistently identified in previous experiments, results appearing dependent upon the investigative strategy used and the antibody employed (see Results 1: Presence of RAS components in hepatic mitochondria, pages 94-125). If a functional, self-sufficient RAS were to exist within mitochondria, it should contain enzymes generating ang II, a role mostly fulfilled by ACE, the enzyme directly responsible for the generation of ang II from ang I throughout the body. Whilst the normal molecular weight of ACE is 180-195 kDa, initial Western blots consistently identified a 55kDa molecule which stained with an ACE (C-terminal) antibody in mitochondrial (particularly CM) fractions. This might represent a finding of genuine biological significance, since shorter natural human and rat ACE isoforms exist [ACE-T, Uniprot P47820], and a novel, specific, truncated intra-mitochondrial form of renin has previously been described (Clausmeyer et al., 2000, Clausmeyer et al., 1999, Wanka et al., 2009). To confirm the identity of the 55 kDa mitochondrial band, a series of experiments were performed to isolate, purify and concentrate the 55 kDa protein and allow proteomic analysis by mass spectrometry.

R2.2 Methods

Immunoprecipitation (IP) was performed on rat liver crude mitochondrial sub-fractions to isolate and concentrate the 55 kDa form of ACE for proteomic analysis by mass spectrometry (for further information see 'M9. Mass Spectrometry'; page 87). Each immunoprecipitation experiment was performed utilising ACE (C-terminal) antibody (Santa Cruz Biotechnology; Dallas, Texas, USA) and the results of immunoprecipitation were assessed by Western blotting or direct silver staining of a Bis-Tris gel following electrophoresis. Each experiment was performed at least three times to ensure consistency of results and representative images are presented below.

R2.2.a. Western blotting & Silver Staining

Western blotting and silver staining utilising the end product (eluate) from immunoprecipitation of a CM fraction with the ACE (C-terminal) antibody demonstrated consistent enrichment of a 50 kDa band, as demonstrated by dense staining and reduced non-specific staining around the band of interest (see Figure R2.1). This represented a consistent 5 kDa reduction in mass, as compared to staining of the CM fraction with ACE C-terminal antibody, which consistently revealed a 55 kDa band, potentially consistent with concentration and removal of contaminants (purification). Multiple copies of the 50 kDa band (containing the protein of interest bound to antibody) were excised from gels to undergo further processing and proteomic analysis by mass spectrometry (see Figure R2.2).

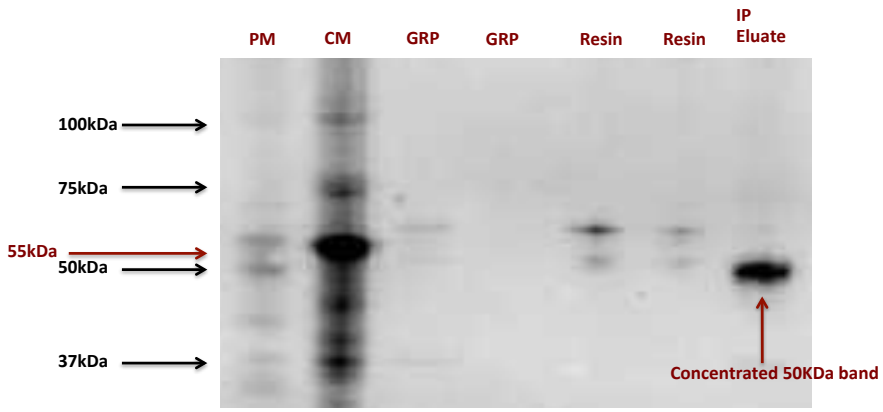

Figure R2.1. Western blot image demonstrating concentration of a 50 kDa band following immunoprecipitation of a crude mitochondrial fraction from rat liver tissue utilising ACE (C-terminal) antibody (IP eluate). When compared to the 55 kDa band seen after electrophoresis of a CM fraction alone, the concentrated band has reduced mass (50 kDa) and reduced non-specific staining (PM- Pure mitochondria; CM- Crude mitochondria; GRP- Control utilising antibody to GRP75; Resin- Control utilising resin only and no antibody; IP Eluate- Eluate from immunoprecipitation of CM with ACE (C-terminal) antibody).

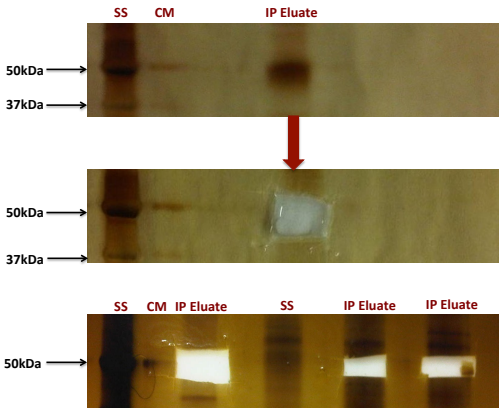

Figure R2.2. Silver stain images again demonstrate concentration of a 50 kDa band (IP Eluate) following immunoprecipitation of a crude mitochondrial fraction from rat liver tissue, utilising ACE (C-terminal) antibody (upper gel). The band is subsequently excised for trypsin digestion and proteomic analysis by mass spectrometry (middle gel). Ultimately multiple bands were processed and excised to increase the volume of protein for mass spectrometry (lower gel) (CM- Crude mitochondria; SS-Size standards; IP Eluate- Eluate from immunoprecipitation of CM fraction with ACE (C-terminal) antibody).

R2.2.b Deglycosylation

Deglycosylation of the IP eluate (of a CM fraction and ACE (C-terminal) antibody) was also performed to remove N-linked glycans from the IP eluate under investigation to compare to the results following IP alone. This technique reduced the heterogeneity of both mass and charge within the sample, thus assisting analysis by mass spectrometry. Western blotting and silver staining were again subsequently performed to assess the effect of deglycosylation and compare to IP alone. Each experiment was performed at least three times to ensure consistency of results and representative images are presented below.

R2.2.c Mass Spectrometry

Following electrophoresis, deglycosylation and excision of the bands of interest, gel-pieces were delivered for mass spectrometry to be performed by experienced technical staff at one of two centres (University College London, UK or Harvard Medical School, Boston, USA (see Methods, page 87 for further information on methodology). Resultant mass spectrometry data were compared against a database of known proteins including UniProtKB/Swiss-Prot, a non-redundant protein sequence database maintained by the UniProt consortium (www.uniprot.org/).

R2.3 Results

R2.3.a. Western Blotting & Silver Staining

Western blotting and silver staining, utilising the deglycosylated IP eluate (from immunoprecipitation of a rat liver CM fraction with ACE (C-terminal) antibody) demonstrated a consistent reduction in mass from 50 kDa to 30 kDa (see Figures R2.3-2.4). Such a finding may be consistent with deglycosylation of a heavily glycosylated glycoprotein, such as ACE.

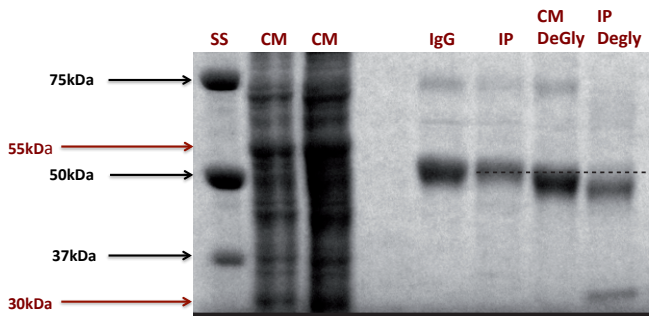

Figure R2.3. Silver stain images again demonstrated concentration of the 50 kDa band in an IP eluate (IP) following immunoprecipitation of a crude mitochondrial fraction from rat liver tissue, utilising ACE (C-terminal) antibody. A crude mitochondrial sub-fraction (CM; also shown for comparison) demonstrated the previously identified 55 kDa band. When the IP eluate underwent deglycosylation (IP Degly), a further 30 kDa band was also seen in addition to the 50 kDa band; however, when the crude mitochondrial sub-fraction underwent deglycosylation, only the 50 kDa band was seen (SS- Size standards; CM-Crude mitochondria; IgG- IgG control (immunoprecipitation of ACE (C-terminal) antibody alone); IP- Eluate from immunoprecipitation of CM fraction with ACE (C-terminal) antibody; CM Degly-Deglycosylation of crude mitochondrial sub-fraction; IP Degly- Deglycosylation of IP eluate).

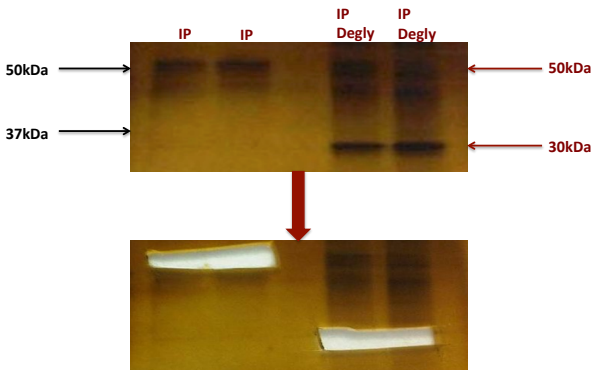

Figure R2.4. Silver stain images again demonstrated concentration of a 30 kDa band (IP Degly) when the IP eluate was deglycosylated and the 50 kDa band (IP) following immunoprecipitation alone (upper gel). Both band sizes (50 kDa and 30 kDa) were carefully excised for proteomic analysis by mass spectrometry (lower gel) (IP-Eluate from immunoprecipitation with ACE (C-terminal antibody); IP Degly-Deglycosylation of eluate from immunoprecipitation).

R2.3.b Mass Spectrometry

Results are presented depending upon the site of analysis. UCL results were analysed via the Protein Lynx Global Server (PLGS) (Waters; Milford, MA, USA) and a 'PLGS score' assigned based upon a computer software algorithm that utilised filtering and scoring routines to analyse all available mass spectrometry data and statistically measure peptide assignment accuracy as well as minimising the occurrence of false positive results (scores greater than 78 are significant ($p<0.05$)). Harvard results were analysed by Seaquest (Thermo Scientific; Waltham, MA, USA), a mass spectrometry data analysis programme that assigns scores such as 'XCorr', a cross-correlation score derived from the database search, which is used to produce the final ranking of candidate peptides in the search (values above 2 represent a good correlation); 'Delta correlation score' (dCn), a score computed by the Seaquest algorithm, which represents an assessment of the distance between the best database search hit and the second best (the closer to 1, the more positive the result); and 'Ions', which is an assessment of how many experimental ions matched with theoretical ions for the peptide under analysis (>70 % represents a positive result).

Samples sent for mass spectrometry analysis include:

i) a 55 kDa band following electrophoresis of a rat liver crude mitochondrial sub-fraction (CM) stained with ACE (C-terminal antibody);
ii) a 50 kDa band following immunoprecipitation of a CM sub-fraction with ACE (C-terminal antibody) (IP); and
iii) a 30 kDa band following deglycosylation of a 50 kDa IP eluate (IP Degly).

i. Rat liver crude mitochondrial sub-fraction (CM 55 kDa band)

Database searches following mass spectrometry of the 55 kDa band derived from crude mitochondrial sub-fraction incubation with ACE (C-terminal) antibody demonstrated no results consistent with ACE protein (see Figure R2.5 and Appendix 3, page 221 for full results). Proteins identified included keratin and dermicidin (likely human contaminants), as well as reagents used during experimentation, such as trypsin (for in-gel protein digestion) and enolase (protein used to standardise mass spectrometry performance). Actual rat liver mitochondrial proteins with similar molecular weight, which could give rise to the non-specific binding of the antibody might include the pyruvate kinase isozymes M1 and M2, and tubulin alpha 8 chain, which has previously been shown to be associated with mitochondria.

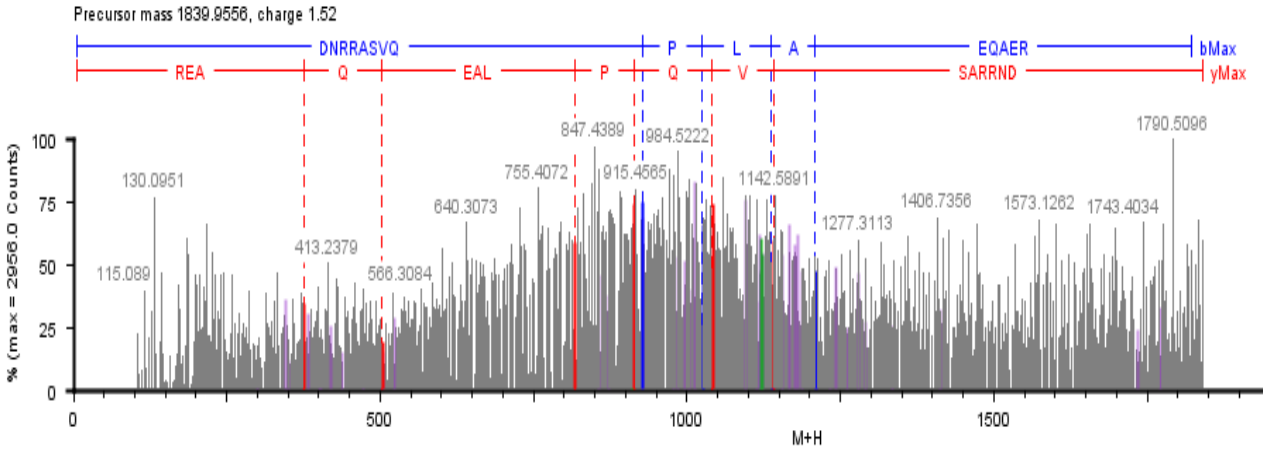

Figure R2.5. Representative mass spectra results, following analysis of a gel piece containing a 55 kDa band derived from a crude mitochondrial sub-fraction of rat liver tissue, demonstrate no findings consistent with ACE.

ii. Immunoprecipitation eluate (IP 50 kDa band)

Database searches following mass spectrometry of the 50 kDa band derived from the immunoprecipitation eluate (of crude mitochondrial sub-fraction incubation with ACE (C-terminal) antibody) similarly demonstrated contaminants and experimental reagents but no results consistent with ACE protein (see Figure R2.6 and Appendix 3, page 221). Two gel-pieces analysed at University College London identified a weak correlation (PLGS scores 76 and 63) of the 50 kDa band with an unknown rat protein of molecular weight approximately 46 kDa.

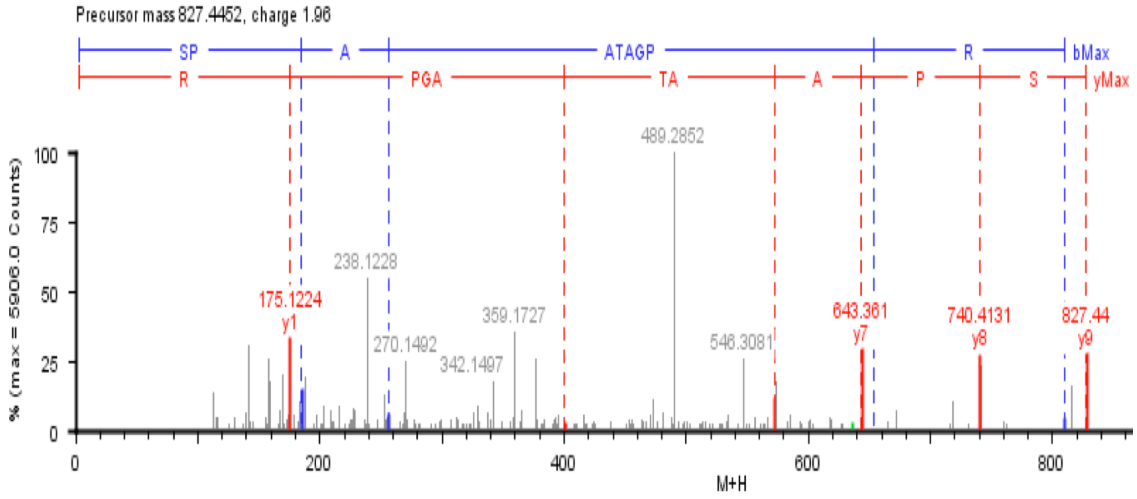

Figure R2.6. Representative mass spectra results after analysis of a gel piece containing a 50 kDa band derived from an immunoprecipitation eluate of a rat liver crude mitochondrial sub-fraction demonstrated no findings consistent with ACE.

iii. Deglycosylated immunoprecipitation eluate (IP Degly 30kDa band)

Database searches following mass spectrometry of the 30 kDa band derived from deglycosylation of the immunoprecipitation eluate of a crude mitochondrial sub-fraction incubated with ACE (C-terminal) antibody demonstrated contaminants (enolase) but no results consistent with ACE protein (see Appendix 3, page 221, for full results).

R2.4 Discussion

Immunoprecipitation of rat liver tissue and ACE (C-terminal) antibody demonstrated consistent concentration of a 50 kDa band, whereas deglycosylation of this immunoprecipitation eluate demonstrated a consistent reduction in mass from 50 kDa to 30 kDa. Database searches following mass spectrometry of 55 kDa (from CM sub-fraction), 50 kDa (from IP eluate) and 30 kDa (from deglycosylated IP eluate) bands demonstrated no results consistent with ACE protein. Proteins consistently identified included contaminants (e.g. keratin and dermicidin), and experimental reagents (e.g. enolase). The results therefore indicate that the 55 kDa band is most likely to represent non-specific binding by the ACE (C-terminal) antibody and although these results do not completely exclude the presence of ACE in low abundance, they do render the presence of ACE in the mitochondria unlikely.

Smaller ACE molecules than the usual size of 170-195 kDa have been previously described with at least four isoforms identified in rats (intracellular 130 and 68 kDa forms and secretory 130 and 60 kDa (Camargo de Andrade et al., 2006). Truncated intra-mitochondrial renin proteins have also been previously described (Wanka et al., 2009, Clausmeyer et al., 2000, Clausmeyer et al., 1999), reinforcing the idea that a truncated mitochondrial ACE protein is possible. However, the truncated renin described lacks a small portion of its overall structure (representing its ER targeting sequence); whereas, a molecular weight of 30-55 kDa is significantly below the usual size of ACE and would necessitate considerable cleavage and processing of the ACE molecule prior to mitochondrial import, potentially rendering its enzymatic activity non-functional and raising the question of what its function may subsequently be. Positive mass spectrometry results here would have suggested a role for a truncated intra-mitochondrial ACE and would therefore imply the existence of a mitochondrial RAS where nuclear genome-derived RAS components are formed within the cytoplasm, subsequently undergoing significant post-translational processing and cleavage prior to mitochondrial import, where intra-mitochondrial conversion of ang I to ang II may take place. Negative mass spectrometry results, as presented here, do not completely exclude the presence of a mitochondrial RAS, but would suggest that if such a system existed, intra-mitochondrial ang I to II conversion would occur via the enzymatic action of non-ACE, alternate, previously undescribed enzymes.

RESULTS 3:

FUNCTIONAL ANALYSIS OF A HEPATIC MITOCHONDRIAL RAS

Abstract

Introduction: Earlier results suggest that mitochondria may contain classical RAS components, although a mitochondrial-specific form of ACE is unlikely to exist. If such a hepatic intra-mitochondrial RAS were to exist, its function remains unknown. To further investigate the potential functions of a mitochondrial RAS, a series of experiments were performed to assess the effects of stimulating or antagonising the renin-angiotensin system.

Methods: Functional fluorescence microscopy was used to measure real-time, dynamic, live-cell (HeLa and HepG2) alterations in multiple, crucial intracellular processes (including mitochondrial membrane potential, intracellular calcium concentration and NADH concentration) following addition of various stimulatory (ang II) and inhibitory factors (Losartan (AT_1R antagonist), PD123319 (AT_2R antagonist) (at both 1 mM and 1 μM), as well as substrates known to alter mitochondrial metabolism in a predictable fashion (including rotenone (inhibitor of complex I & II), FCCP (uncoupling agent) and oligomycin (inhibitor of ATP synthase)). Real-time, dynamic alterations in oxygen consumption were also assessed via a Clark electrode in response to angiotensin II and its receptor antagonists, as well as established mitochondrial substrates.

Results: Functional fluorescence microscopy data demonstrated accurate use of positive controls with FCCP decreasing mitochondrial membrane potential, intracellular calcium and intracellular NADH; and rotenone resulting in significant decreases in mitochondrial membrane potential and intracellular calcium, associated with an increase in NADH concentration. However, ang II, losartan and PD123319 addition led to inconsistent results without biological plausibility in both HeLa and HepG2 cells.

The addition of reagents to live HepG2 cells demonstrated consistent oxygen consumption results: ang II had no significant effect upon cellular oxygen consumption at 1 μM or 1 mM; whereas, both Losartan and PD123319 had no effect at 1 μM concentration but led to a significant increase in cellular oxygen consumption at 1 mM concentration. When added to live HeLa cells, ang II led to a significant decrease in cellular oxygen consumption at 1 μM concentration; whereas, both Losartan and PD123319 had no significant effect at a concentration of 1 μM, but led to a significant increase in cellular oxygen consumption at 1 mM concentration.

Discussion: It can be hypothesized that the HepG2 results presented may be inconsistent due to the dual presence of cell membrane and mitochondrial membrane receptors; however, results derived from HeLa cells should represent effects at the mitochondrial membrane only (as HeLa cells contain no plasma membrane ang receptors). Thus, addition of ang II may lead to a decrease in cellular oxygen consumption, a result that would be consistent with previously described studies but this effect is likely to be non-specific in that it can be prevented by both AT_1 and AT_2 receptor inhibitors. Further, the physiological intracellular concentration of ang II is likely to be in the picomolar range, in accordance with the sub-nanomolar affinity of angiotensin receptors on the cell surface, and the supra-physiological reagent concentrations utilised here may therefore result in non-endogenous effects.

R3.1 Introduction

Previous Western blot, immunohistochemistry and electron microscopy results suggested that mitochondria may contain classical RAS components, although a mitochondrial-specific form of ACE is unlikely to exist. Specifically, Western blotting demonstrated renin, AT_1R, AT_2R and angiotensin within sub-fractionated mitochondrial compartments in both rat liver tissue and HepG2 cells; whilst ACE was absent from all mitochondrial sub-fractions in both rat liver tissue and HepG2 cells at its usual size of 170-180 kDa. Immunofluorescence and confocal microscopy of HepG2 cells demonstrated the presence of renin, ang and ACE within mitochondria, whilst immunogold labeling and electron microscopy demonstrated ACE in association with rat liver mitochondria and mitochondria of the HepG2 cell line. However, mass spectrometry results indicated that a truncated ACE of 55 kDa molecular weight was not present within mitochondria.

If a hepatic intra-mitochondrial RAS were to exist, its function remains unknown. Abadir *et al* (2011) suggest that activation of a mitochondrial RAS is coupled to mitochondrial nitric oxide production and can modulate cellular respiration, whilst intracrine hepatic RAS activation may be pro-inflammatory and pro-fibrotic, contributing to the pathogenesis of chronic liver disease and liver fibrosis/cirrhosis (Grace et al., 2012). To further investigate the potential functions of a mitochondrial RAS, a series of experiments were performed to assess the mitochondrial effects of stimulating or antagonising the renin-angiotensin system. A variety of crucial mitochondrial functions were therefore assessed, including:

i. NADH concentration: NADH is an energy-rich molecule produced within the mitochondrial matrix via the Krebs cycle. The redox energy from such molecules is transferred to oxygen in several steps via the electron transport chain within the inner mitochondrial membrane.
ii. Mitochondrial membrane potential ($\Delta\Psi m$; for further information see ‘M1. Materials’; page 61): The redox action of NADH allows the generation of energy utilised to pump protons into the mitochondrial intermembrane space, forming an electrochemical proton gradient or mitochondrial membrane potential. Protons can only subsequently re-enter the mitochondrial matrix via the ATP synthase complex, where their potential energy is employed to drive the generation of

chemical energy in the form of ATP. Thus the maintenance and stability of this membrane potential remains crucial for fundamental mitochondrial function and a significant loss of $\Delta\Psi m$ renders the cell depleted of energy with subsequent cell death.

iii. Oxygen consumption: Mitochondria utilise oxygen to produce ATP and the rate of oxygen consumption is therefore a valuable tool in the assessment of mitochondrial function as it directly reflects the efficiency of oxidative phosphorylation.

iv. Intracellular calcium concentration (for further information see ‘M1. Materials’; page 61): Free intracellular calcium concentrations are vital for the regulation of an array of cellular reactions and signal transduction; and mitochondria can have significant effects on calcium levels via interaction with endoplasmic reticulum (ER), the most significant site of intracellular calcium storage.

As in previous experiments, the HepG2 cell line and rat liver tissue were utilised. HeLa cells, which lack plasma membrane angiotensin receptors [personal communication: Peter Varnai (Semmelweis Medical School, Budapest, Hungary)] were also used, as any cellular metabolic alteration identified following the introduction of RAS components must occur secondary to direct mitochondrial or non-specific action only, and not via plasma membrane angiotensin receptors.

R3.2 Methods

R3.2.a. Functional Fluorescence Microscopy

Functional fluorescence microscopy refers to the process of imaging live cells on a confocal microscope, following the addition of fluorescent molecules. It is used to measure real-time, dynamic alterations in multiple intracellular processes, in response to various stimulatory or inhibitory factors. The intensity of fluorescence dynamically alters as the metabolic process under examination changes, allowing quantitative study of cellular pathways within live cells.

The actual mitochondrial membrane potential of hepatocytes has been estimated as -154.3 mV in young Fischer rats (Hagen et al., 1997); however, functional fluorescence microscopy allows quantitative assessment of changes in mitochondrial membrane potential (as well as other parameters), in response to various internal and external factors, in a consistent and reproducible manner (see Figures R3.1-R3.2 and 'M1. Materials'; page 61). Here, changes in mitochondrial membrane potential (TMRM; excitation at 500 nm), intracellular calcium concentration (Fluo 4; excitation at 488 nm) and NADH concentration (auto-fluorescence; excitation at 364 nm) were assessed in response to ang II agonism and antagonism (Losartan, AT_1R antagonist and PD123319, AT_2R antagonist), and to well-established substrates that alter mitochondrial metabolism in a predictable fashion, including rotenone (inhibitor of electron transport chain complex I & II) and FCCP (uncoupling agent). Histamine was also added as a positive control, as binding to Histamine receptors on the plasma membrane should cause a cytosolic, and subsequent, mitochondrial Ca^{2+} signal.

Live cells were allowed to equilibrate for at least five minutes within the microscope, on a heated stage at 38 °C. Cellular fluorescence was constantly measured during the experiment, utilsiing statistical software pre-installed on the microscope, and the initial fluorescence at the commencement of the experiment was arbitrarily set to a relative fluorescence value of 1. Relative changes in fluorescence were subsequently assessed as reagents were added to the live cells (with relative fluorescence increasing >1 for brighter fluorescence and decreasing <1 for a reduction in fluorescence, as compared to the original fluorescence at the commencement of the experiment). Relative fluorescence was measured for at least two

minutes before and after addition of each of the various reagents. Each experiment was carried out at least five times and a mean of the alteration in relative fluorescence for all experiments calculated and presented below. Statistically significant alterations in mean relative fluorescence, before and after addition of various substrates, were assessed by two-tailed t-test; and a probability value of less than 5 % ($p < 0.05$) was considered significant.

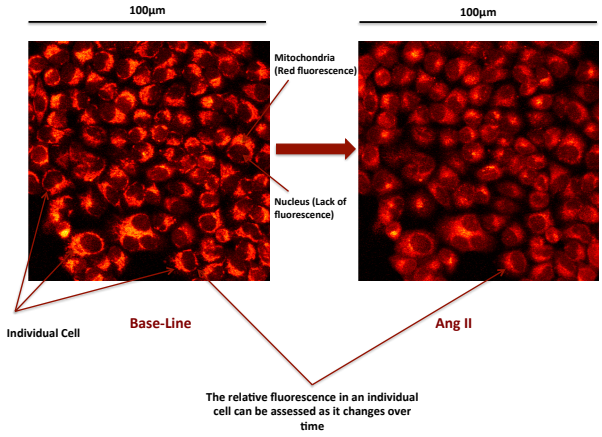

Figure R3.1. Representative image of a decrease in mitochondrial membrane potential, seen as a decrease in relative fluorescence (due to pre-incubation with TMRM) between left and right images. The change occurred following addition of ang II (1mM) and these example images demonstrate that alterations in relative fluorescence can be visibly observed with the naked eye, as well as formally quantified.

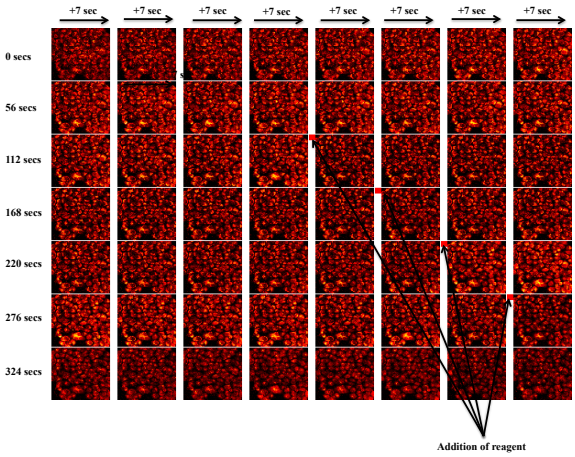

Figure R3.2. Representative image grid demonstrating relative increases and decreases in red fluorescence (mitochondrial membrane potential) in live HeLa cells over time (approx.7 minutes), following addition of various substrates (a red square in the top right hand corner of an image demonstrates that a new reagent has been added). The grid of images is read from left to right and a new image is captured every seven seconds.

R3.2.b. Oxygen Consumption

Real-time, dynamic alterations in oxygen consumption were assessed via a Clark electrode (see Figure 3.14 and 'M10. Oxygen Consumption'; page 92), in both HepG2 and HeLa cells, in response to angiotensin II and to its receptor antagonists (Losartan and PD123319). Well-established substrates that alter mitochondrial metabolism in a predictable fashion, including oligomycin (inhibitor of ATP synthase) and FCCP (uncoupling agent) were utilised as positive controls (for further information see 'M1. b. Reagents'; page 61).

Two million live cells (HepG2 or HeLa) were allowed to equilibrate within the chamber of the Clark electrode at 38 °C and oxygen consumption set to 100 % after an equilibration period of at least five minutes. The rate of oxygen consumption was subsequently measured for two minutes and a basal rate of oxygen consumption measured and set arbitrarily to 100 %. Thus, any subsequent change in the rate of oxygen consumption per minute was expressed as a percentage change from 100 % (e.g. -0.02 equals decrease in rate of oxygen consumption of 2 % per minute from pre-set level of 100 %). A substrate was subsequently added via the designated hole in the lid of the apparatus chamber and the rate of oxygen consumption again measured for two minutes. The rate of oxygen consumption naturally reduces over time in cells examined in this fashion and each separate set of cells was therefore replaced after ten minutes to allow for this. Each experiment was performed five times and the mean of the results presented below. Statistically significant alterations in mean rates of oxygen consumption, following addition of various substrates, were assessed by two-tailed t-test; and a probability value of less than 5 % ($p<0.05$) was considered significant.

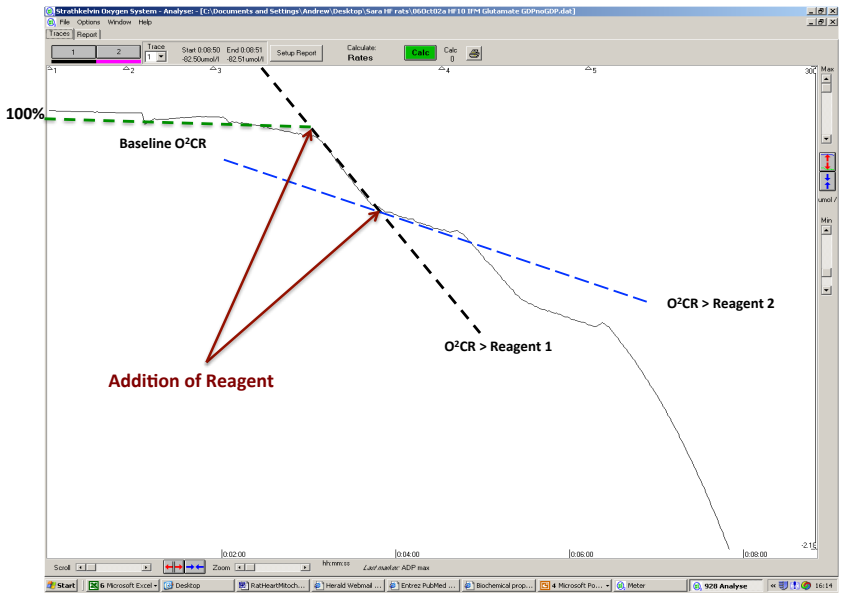

Figure R3.14. An image of the computer software utilised to measure oxygen consumption demonstratedd how alterations in the rate of oxygen consumption were calculated. The initial level is arbitrarily set to 100 % after cellular equilibration (for at least five minutes) and the steepness of the rate of decline was measured following addition of various reagents. Oxygen consumption was assessed for at least two minutes prior to and following the addition of each reagent (O^2CR- Rate of oxygen consumption).

R3.3 Results

R3.3. a. Functional Fluorescence Microscopy

R3.3. a.i HepG2 Cells (see Figures R3.3-3.6)

The addition of FCCP to live HepG2 cells led to predictable and significant decreases in intracellular calcium, NADH and mitochondrial membrane potential. Ang II addition produced a significant decrease in NADH concentration and a significant increase in mitochondrial membrane potential, with no significant effect noted upon intracellular calcium. Losartan addition significantly decreased intracellular NADH and significantly increased intracellular calcium levels; whereas, PD123319 treatment had no significant effects.

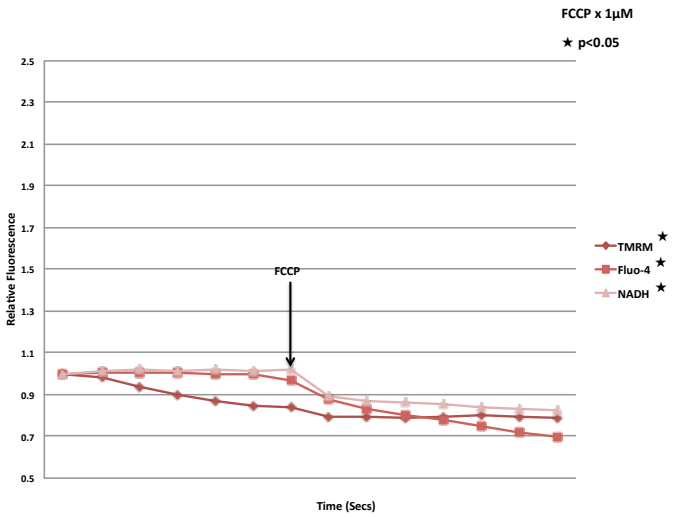

Figure R3.3. The addition of FCCP (uncoupling agent; concentration 1 µM) to live HepG2 cells led to predictable and significant decreases in concentrations of intracellular calcium (Fluo-4; p= 0.000) and NADH (p= 0.000), as well as a significant decrease in mitochondrial membrane potential (TMRM; p= 0.006). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

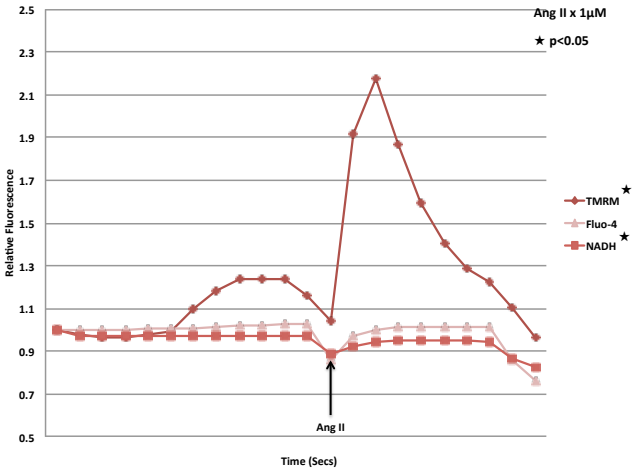

Figure R3.4. The addition of angiotensin II (concentration 1 μM) to live HepG2 cells led to a significant decrease in concentrations of NADH (p= 0.020) and a significant increase in mitochondrial membrane potential (TMRM; p= 0.036). There was no effect upon intracellular calcium (Fluo-4; p= 0.223). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

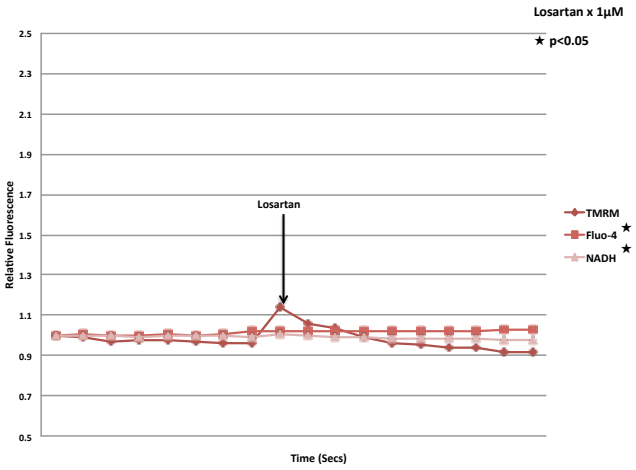

Figure R3.5. The addition of Losartan (AT_1R antagonist; concentration 1 μM) to live HepG2 cells led to a significant decrease in intracellular NADH (p= 0.000), and a significant increase in intracellular calcium levels (Fluo-4; p= 0.000). No significant effect was seen upon mitochondrial membrane potential (TMRM; p= 0.927). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

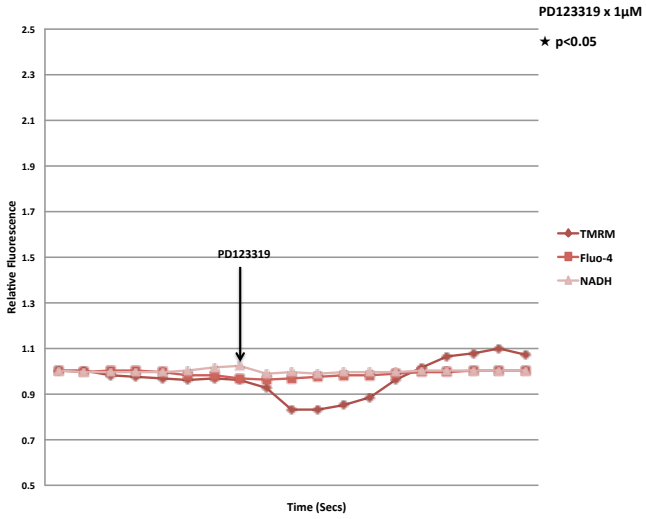

Figure R3.6. The addition of PD123319 (AT_2R antagonist; concentration 1 μM) to live HepG2 cells had no significant effects upon intracellular calcium concentration (Fluo-4; p= 0.734), NADH concentration (p= 0.431) or mitochondrial membrane potential (TMRM; p= 0.918. Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

R3.3. a.ii HeLa Cells (see Figures R3.7-3.13)

HeLa cells have no plasma membrane angiotensin receptors [personal communication: Peter Varnai (Semmelweis Medical School, Budapest, Hungary)] and therefore any cellular metabolic alteration following the introduction of RAS components must occur secondary to direct mitochondrial or non-specific action only, and not via plasma angiotensin receptors. The addition of angiotensin II to live HeLa cells led to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration. Losartan addition conversely produced a significant decrease in intracellular calcium concentration and a significant increase in mitochondrial membrane potential, and PD123319 resulted in significant decreases in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration.

Control reagents had predictable effects, with histamine causing no effects apart from a brief calcium surge (calcium spike); and FCCP leading to a significant decrease in mitochondrial membrane potential, intracellular calcium and intracellular NADH. Rotenone addition resulted in a significant decrease in mitochondrial membrane potential, and intracellular calcium, associated with an increase in NADH concentration; and when HeLa cells were pre-treated with rotenone prior to ang II addition, ang II led to an additional increase in mitochondrial depolarization and NADH levels, super-imposed upon rotenone effects, suggesting that the mechanism of ang II action was separate to that of rotenone (therefore not occurring via complex I).

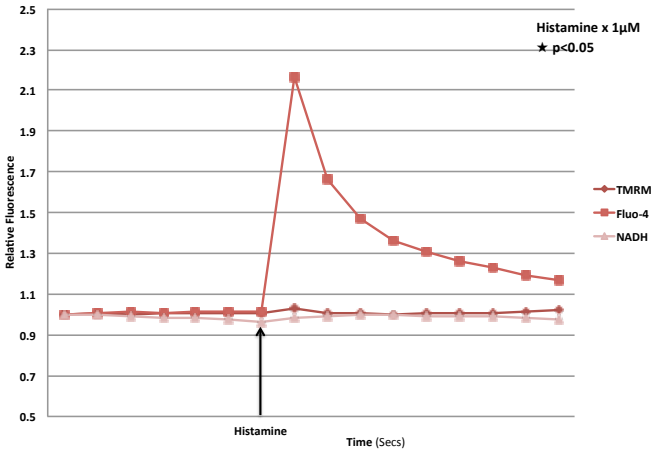

Figure R3.7. The addition of Histamine (concentration 1 μM) to live HeLa cells led to a predictable and swift increase in intracellular calcium levels (calcium spike) (Fluo-4) but no longer-term effects upon intracellular calcium levels (Fluo-4; p= 0.349), mitochondrial membrane potential (TMRM; p= 0.524), or NADH concentration (p= 0.194). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

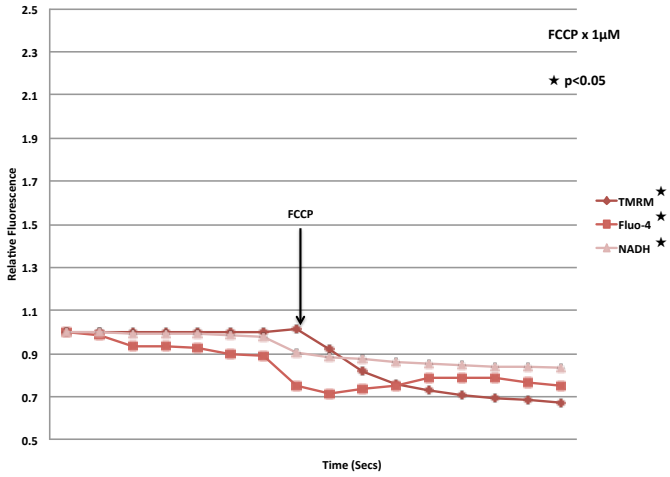

Figure R3.8. The addition of FCCP (uncoupling agent; concentration 1 μM) to live HeLa cells led to a predictable and significant decrease in concentrations of intracellular calcium (Fluo-4; p= 0.002) and NADH (p= 0.000), as well as a significant decrease in mitochondrial membrane potential (TMRM; p= 0.000). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

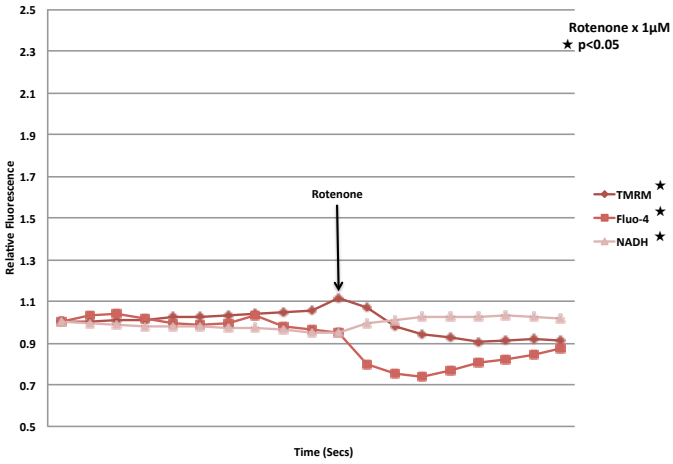

Figure R3.9. The addition of rotenone (inhibitor of electron transport chain complex I & II; concentration 1 μm) to live hela cells led to a predictable and significant decrease in mitochondrial membrane potential (TMRM; p= 0.000), and intracellular calcium (Fluo-4; p= 0.000), associated with an increase in NADH concentration (p= 0.000). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

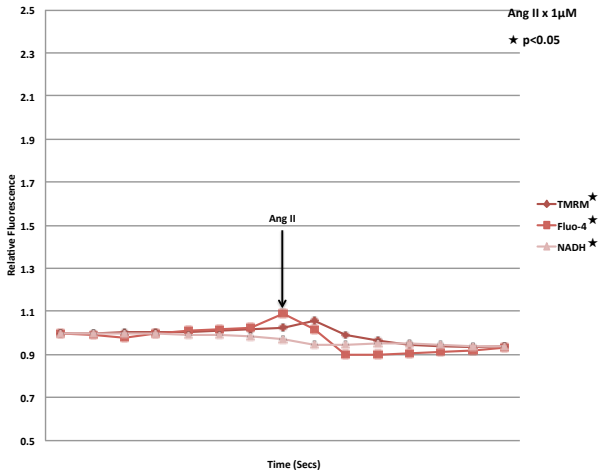

Figure R3.10. The addition of angiotensin II (concentration 1 μM) to live HeLa cells led to a significant decrease in mitochondrial membrane potential (TMRM; p= 0.000), intracellular calcium concentration (Fluo-4; p= 0.011) and NADH (p= 0.000) concentration. Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

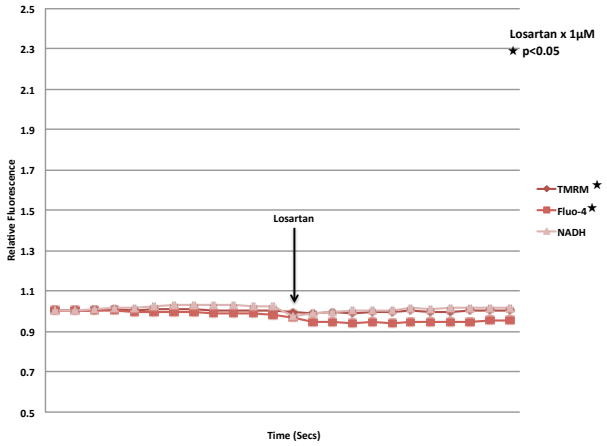

Figure R3.11. The addition of Losartan (AT_1R antagonist; concentration 1 μM) to live HeLa cells led to a significant decrease in intracellular calcium concentration (Fluo-4; p= 0.000) and a significant increase in mitochondrial membrane potential (TMRM; p= 0.005) with no significant effect upon NADH concentration (p= 0.140). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

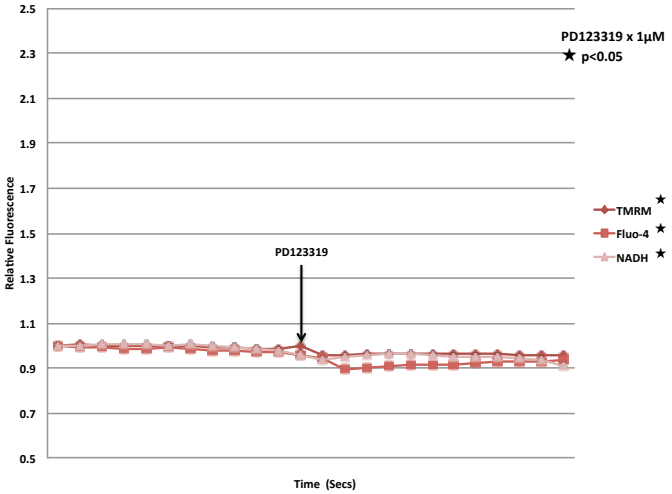

Figure R3.12. The addition of PD123319 (AT_2R antagonist; concentration 1 μM) to live HeLa cells led to significant decreases in mitochondrial membrane potential (TMRM; p= 0.000), intracellular calcium concentration (Fluo-4; p= 0.000) and NADH concentration (p= 0.000). Each time point represents 7 seconds and each experiment was performed 6 times with the mean presented here.

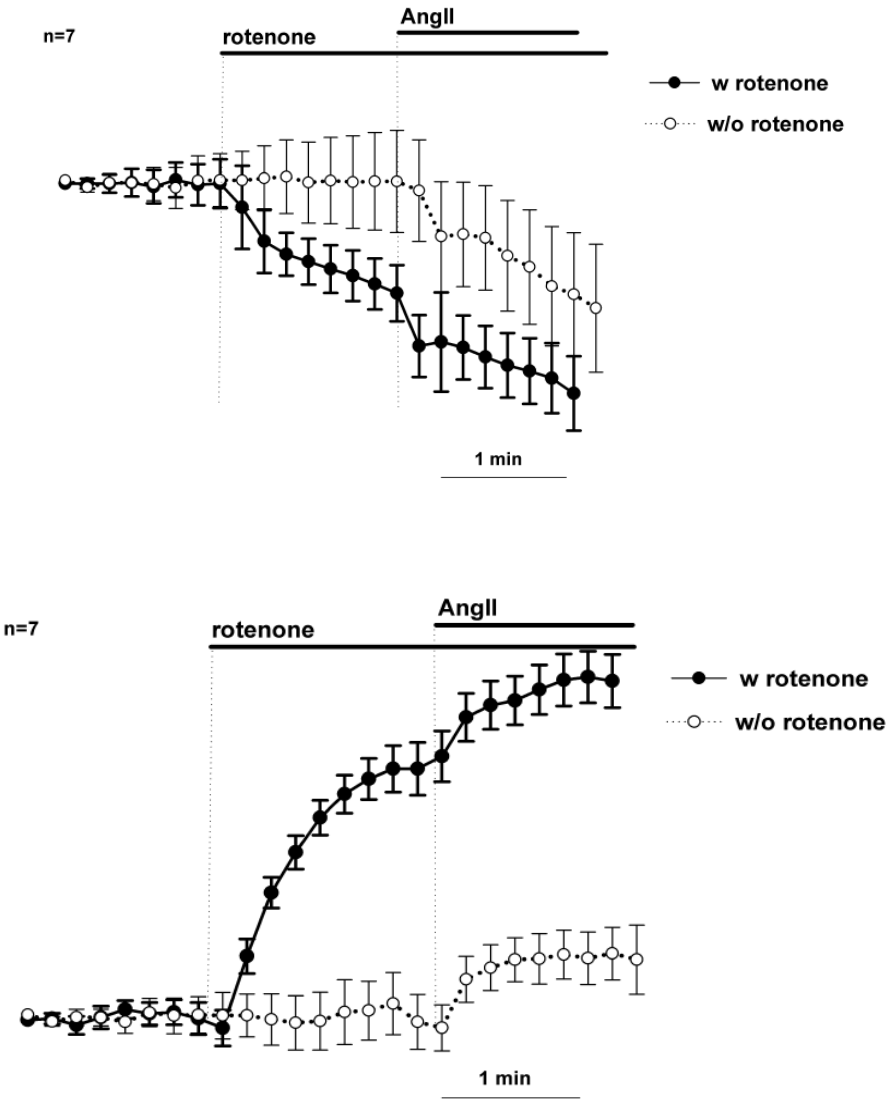

Figure 3.13.a. and 3.13.b. Addition of ang II to HeLa cells pre-treated with rotenone demonstrated additional mitochondrial depolarization (a. Upper image) and NADH elevation (b. Lower image), super-imposed upon the effects occurring secondary to rotenone (w rotenone- Pre-treated with rotenone; w/o rotenone- Not pre-treated with rotenone).

Reagent	Cell-Line	NADH	Calcium	Mitochondrial Membrane Potential
Ang II (1 µm)				
	HepG2	**Decrease**	**Nil**	**Increase**
	HeLa	**Decrease**	**Decrease**	**Decrease**
Losartan (1 µm)				
	HepG2	**Decrease**	**Increase**	**Nil**
	HeLa	**Nil**	**Decrease**	**Increase**
PD123319 (1 µm)				
	HepG2	**Nil**	**Nil**	**Nil**
	HeLa	**Decrease**	**Decrease**	**Decrease**

Table R3.3. A summary of data derived from the addition of ang II or RAS antagonists during fluorescence microscopy experimentation in live HepG2 and HeLa cells.

R3.3.b Oxygen Consumption

R3.3. b.i. HepG2 Cells (see Figures R3.15-R3.18)

These experiments demonstrated that there was no effect upon mitochondrial oxygen consumption, as measured by Clark electrode, of adding angiotensin II (1 μM or 1 mM) to live HepG2 cells. Similarly, there was no effect upon adding RAS antagonists at 1 μM; however, when the concentration was increased to 1 mM, an increase in the rate of oxygen consumption was seen following addition of both Losartan and PD123319; however, such a concentration is significantly higher than the concentratyions that would be found *in vivo*.

	n	Mean Basal Rate (AU)	Rate After Reagent (AU)	Mean Change in Rate (AU)	p
Ang II (1μM)	**11**	**-0.230**	**-0.204**	**-0.016**	**0.062**
Ang II (1mM)	**13**	**-0.251**	**-0.249**	**-0.003**	**0.502**
Losartan (1μM)	**8**	**-0.072**	**-0.064**	**-0.002**	**0.375**
Losartan (1mM)	**6**	**-0.137**	**-0.179**	**0.042**	**0.029**
PD123319 (1μM)	**8**	**-0.072**	**-0.079**	**0.002**	**0.533**
PD123319 (1mM)	**6**	**-0.137**	**-0.174**	**0.037**	**0.019**

Table R3.1. Mean rates of oxygen consumption measured at baseline and after addition of each reagent in live HepG2 cells. Rates of oxygen consumption are expressed as a change in oxygen consumption per minute, as compared to 100 % (e.g. -0.02 arbitrary units (AU) equals decrease in rate of oxygen consumption of 2 % per minute from baseline rate of oxygen consumption). Significant changes in rate of oxygen consumption are highlighted in red ($p < 0.05$).

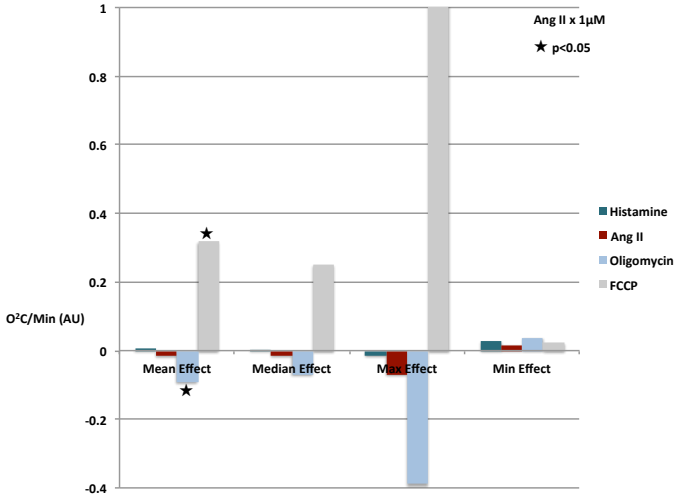

Figure R3.15. The addition of angiotensin II to live HepG2 cells, at a concentration of 1 μM, had no significant effect upon cellular oxygen consumption (p= 0.062). Substrates with known mitochondrial effects (histamine- no effect; (p= 0.307) oligomycin (complex I&II inhibitor)- significant decrease (p= 0.001); FCCP (uncoupling agent)- significant increase (p= 0.000)) were included as positive and negative controls (Ang II- Angiotensin II; O^2C- Change in Oxygen consumption; AU- Arbitrary units).

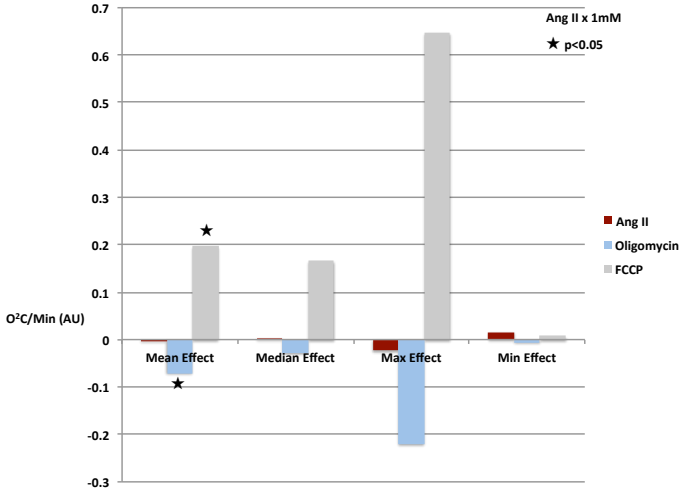

Figure R3.16. The addition of angiotensin II to live HepG2 cells, at a concentration of 1 mM, had no significant effect upon cellular oxygen consumption (p= 0.502). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p= 0.003); FCCP (uncoupling agent)- significant increase (p= 0.002)) were included to act as positive controls.

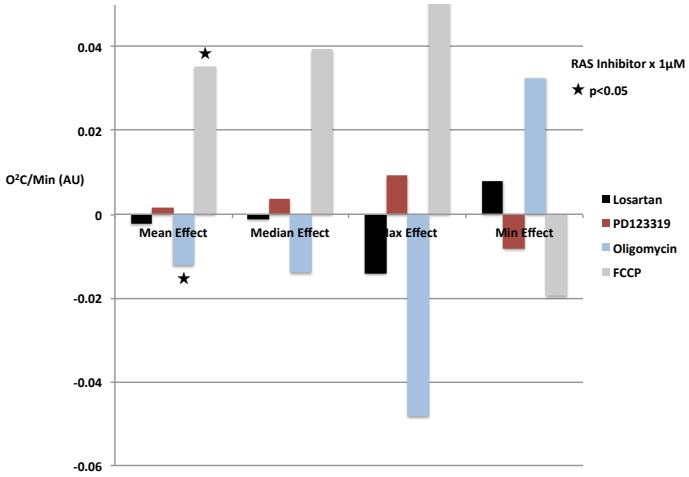

Figure R3.17. The addition of Losartan (AT^1R antagonist) and PD123319 (AT^2R antagonist) to live HepG2 cells, at a concentration of 1 μM, had no significant effect upon cellular oxygen consumption (p= 0.375 and 0.533 respectively). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p= 0.049); FCCP (uncoupling agent)- significant increase (p= 0.001)) were included to act as positive controls.

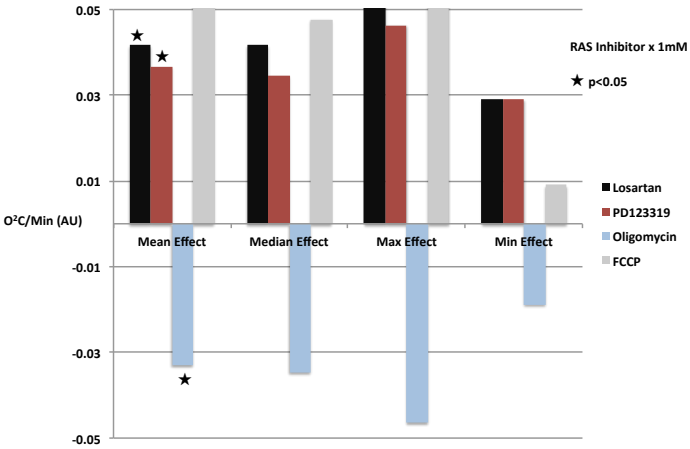

Figure R3.18. The addition of Losartan (AT^1R antagonist) and PD123319 (AT^2R antagonist) to live HepG2 cells, at a concentration of 1 mM, led to a significant increase in cellular oxygen consumption (p= 0.029 and 0.019 respectively). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p= 0.002); FCCP (uncoupling agent)- no change (p= 0.058)) were included to act as positive controls.

R3.3. b.i. HepG2 Cells

These data demonstrate that a significant decrease in mitochondrial oxygen consumption was seen upon addition of angiotensin II (concentration 1 μM) to live HeLa cells, as assessed by Clark electrode (see Table R3.2). There was no effect upon adding RAS inhibitors, unless the concentration was increased to 1 mM, at which point an increase in the rate of oxygen consumption was seen following addition of both Losartan and PD123319 (see Figures R3.19-R3.21); an effect that is biologically inconsistent and occurring at only extremely high antagonist concentrations.

	n	Mean Basal Rate (AU)	Rate After Reagent (AU)	Change in Rate (AU)	p
Ang II (1 μM)	**7**	**-0.485**	**-0.013**	**-0.007**	**0.005**
Losartan (1 μM)	**7**	**-0.039**	**-0.050**	**0.004**	**0.149**
Losartan (1 mM)	**6**	**-0.137**	**-0.179**	**0.0412**	**0.029**
PD123319 (1 μM)	**6**	**-0.039**	**-0.034**	**0.003**	**0.626**
PD123319 (1 mM)	**6**	**-0.137**	**-0.174**	**0.037**	**0.019**

Table R3.2. Mean rates of oxygen consumption measured at baseline and after addition of each reagent in live HeLa cells. Rates of oxygen consumption are expressed as a change in oxygen consumption per minute, as compared to 100 % (e.g. -0.02 arbitrary units (AU) equals decrease in rate of oxygen consumption of 2 % per minute from baseline rate of oxygen consumption). Significant changes in rate of oxygen consumption are highlighted in red.

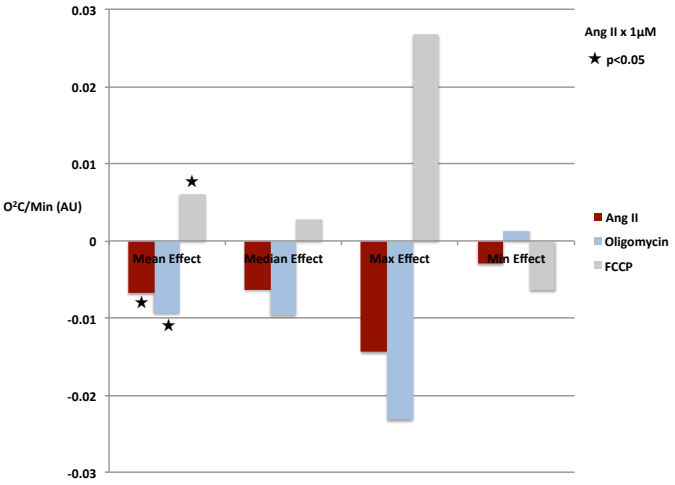

Figure R3.19. The addition of angiotensin II to live HeLa cells, at a concentration of 1 μM, led to a significant decrease in cellular oxygen consumption (p=0.005). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p=0.003); FCCP (uncoupling agent)- significant increase (p=0.005)) were included to act as positive controls.

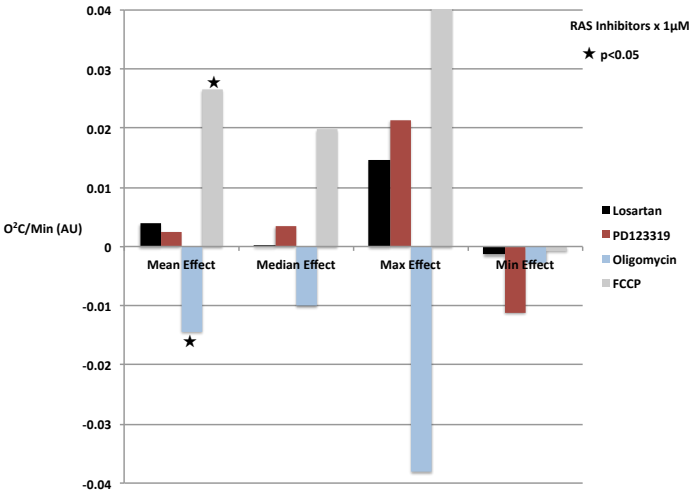

Figure R3.20. The addition of Losartan (AT^1R antagonist) and PD123319 (AT^2R antagonist) to live HeLa cells, at a concentration of 1 μM, had no significant effect upon cellular oxygen consumption (p=0.149 and 0.626 respectively). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p=0.000); FCCP (uncoupling agent)- significant increase (p=0.006)) were included to act as positive controls.

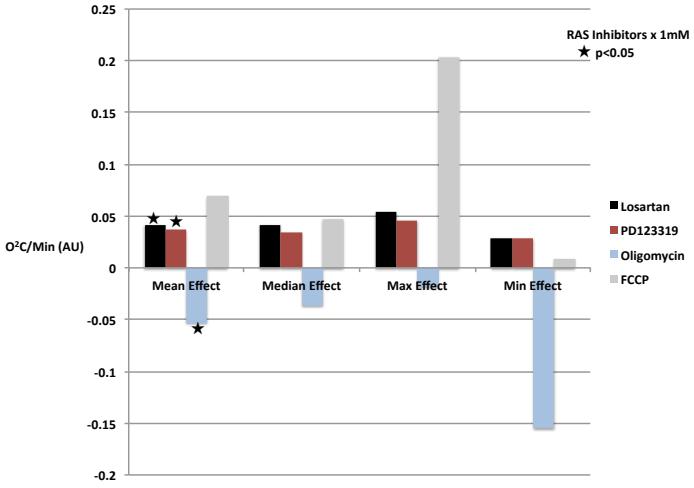

Figure R3.21. The addition of Losartan (AT^1R antagonist) and PD123319 (AT^2R antagonist) to live HeLa cells, at a concentration of 1 mM, led to a significant increase in cellular oxygen consumption (p=0.029 and 0.019 respectively). Substrates with known mitochondrial effects (oligomycin (complex I&II inhibitor)- significant decrease (p=0.002); FCCP (uncoupling agent)- non-significant increase (p=0.058)) were included to act as positive controls.

Reagent	Cell-Line	Concentration of Reagent	Rate of Oxygen Consumption
Ang II	HepG2	1 µM	Nil
		1 mM	Nil
	HeLa	1 µM	Decrease
		1 mM	N/A
Losartan	HepG2	1 µM	Nil
		1 mM	Increase
	HeLa	1 µM	Nil
		1 mM	Increase
PD123319	HepG2	1 µM	Nil
		1 mM	Increase
	HeLa	1 µM	Nil
		1 mM	Increase

Table R3.4. A summary of data derived from the addition of ang II or RAS antagonists during oxygen consumption experiments in live HepG2 and HeLa cells.

R3.4 Discussion

These functional fluorescence microscopy results demonstrate accurate positive controls with the use of histamine resulting in no effects apart from a brief intracellular calcium surge (calcium spike); FCCP leading to significant decreases in mitochondrial membrane potential, intracellular calcium and intracellular NADH; and rotenone resulting in significant decreases in mitochondrial membrane potential, and intracellular calcium, associated with an increase in NADH concentration (see Table R3.3). These control data indicate that the functional fluorescence technique described is an accurate assessment of mitochondrial function in these cell-lines. When the same technique was used and ang II or its antagonists were applied to live HepG2 or HeLa cells, inconsistent results were demonstrated. Ang II (concentration 1 μM) added to live HepG2 cells caused a significant decrease in NADH concentration and a significant increase in mitochondrial membrane potential; Losartan (AT_1R antagonist; concentration 1 μM) led to a significant decrease in intracellular NADH and a significant increase in intracellular calcium levels; and PD123319 (AT_2R antagonist; concentration 1 μM) had no significant effects. In HeLa cells, ang II (concentration 1 μM) led to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration; Losartan (AT_1R antagonist; concentration 1 μM) led to a significant decrease in intracellular calcium concentration and a significant increase in mitochondrial membrane potential; and PD123319 (AT_2R antagonist; concentration 1 μM) led to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration.

Published studies suggest that the addition of ang II might be expected to depolarize the mitochondrial membrane potential (Doughan et al., 2008), and decrease oxygen consumption via AT_1-receptor dependent action (Cassis et al., 2002). Further, ang II might be expected to reduce NADH levels via the activation of plasma membrane-bound NADH-/NADPH-oxidases, contributing to decreased mitochondrial membrane potential and oxygen consumption, as well as increased production of reactive oxygen species and superoxides, effects that are antagonized by AT_1R inhibitors and less so by AT_2R inhibitors (Zhang et al., 1999a). Ang II has also been shown to increase intracellular calcium concentration, similarly via action at the AT_1 receptor (Greco et al., 2002, Nitschke et al., 2000).

The non-consistent results seen in these experiments with HepG2 cells could be ascribed to confusing dual effects of both cell membrane and intra-cellular or mitochondrial (if they exist) angiotensin receptors in HepG2 cells. However, HeLa cell results should represent true mitochondrial effects of reagent addition, without the compromising effects of cell membrane receptors (as they contain no cell-membrane ang receptors), suggesting that the addition of ang II leads to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration via an intracellular mechanism (rather than acting via cell membrane receptors), with NADH and mitochondrial membrane potential effects antagonised by the AT_1R antagonist Losartan and AT_2R antagonists having no effect. These effects are potentially compatible with two hypotheses: ang II might inhibit complex I, thus reducing NADH consumption and depolarising the mitochondrial membrane; or ang II may stimulate mitochondrial metabolism via a non-Ca^{2+} dependent mechanism. In order to test whether the effect of ang II occurs via increased NADH from the Krebs cycle, I pre-treated HeLa cells were pre-treated with rotenone (complex I inhibitor i.e. inhibitor of NADH consumption) to demonstrate that ang II led to further elevations of NADH, even following rotenone application, suggesting a separate mechanism of action. Furthermore, ang II increased mitochondrial depolarisation even in the presence of rotenone, potentially indicating a direct effect upon ATP synthase.

Another potential strategy to investigate such RAS effects upon mitochondria would be to add reagents to isolated mitochondria; however, such techniques have recently fallen out of favour. Isolation of pure mitochondria exposes the mitochondria to non-physiological oxygen conditions and removes cellular regulatory systems, thus, all results are assessed outside the context of the mitochondrion's normal functioning conditions. Further, although the intracellular concentrations of ang II are yet to be reported, tissue ang II levels are likely to vary from picomolar to the low nanomolar range (depending upon tissue type and conditions), and the physiological concentrations of intracellular ang II are likely to occur in the picomolar range, in accordance with the sub-nanomolar affinity of angiotensin receptors on the cell surface. In contrast, isolated mitochondria are inevitably exposed to substrate concentrations far higher than that found in the endogenous cellular microenvironment *in vivo*. To provide the most robust evidence for a functioning mitochondrial RAS, a combination of data from isolated mitochondria and intact cells, in conjunction with both *in vivo* and *in vitro* techniques is likely to be required.

Abadir *et al* (2011) suggested that functional AT_2Rs are present on inner mitochondrial membranes (co-localising with ang II) of rat heart and liver, where they lead to modulation of respiration via coupling to mitochondrial nitric oxide production. However, the AT_2R (although highly expressed in the foetus) has low expression levels in normal adults and its precise role remains open for clarification. The AT_2R may be involved in signaling pathways such as activation of protein phosphatases and the bradykinin/nitric oxide/cyclic guanosine 3',5'-monophosphate pathway, as well as stimulation of phospholipase A_2, but its only true established function is vasodilation, although it may be involved in cardiovascular remodeling and inflammation. Similarly, it may also be involved in apoptotic cell death, thereby playing a role in developmental biology; however, mice lacking the gene encoding the AT_2R develop normally and have a normal blood pressure (although they display an increased response to the addition of ang II) (Hein et al., 1995), potentially suggesting that it may not play a crucial role in mitochondria or elsewhere. Abadir's group also utilised only one antibody for their immunofluorescence results and provided no examination of antibody specificity: their results should therefore be interpreted with this in mind. Further, the study utilised only CGP 42112 for AT_2R agonism, a compound that has been reported to antagonise the AT_1R as well as agonise the AT_2R, and a functional assessment of endogenous ATR ligands was not undertaken.

In contrast to these functional immunofluorescence data, the addition of various reagents to live HepG2 cells demonstrated consistent oxygen consumption results: ang II had no significant effect upon cellular oxygen consumption at 1 μM or 1 mM; whereas, both Losartan (AT^1R antagonist) and PD123319 (AT^2R antagonist) had no effect at 1 μM concentration but led to a significant increase in cellular oxygen consumption at a high 1 mM concentration (see Table R3.4). When added to live HeLa cells, ang II led to a significant decrease in cellular oxygen consumption at 1 μM concentration; whereas, both Losartan and PD123319 had no significant effect at a concentration of 1 μM, but led to a significant increase in cellular oxygen consumption at 1 mM concentration. As previously discussed, the HepG2 cell results may be confused by the dual presence of cell membrane and mitochondrial membrane receptors; whereas, results derived from HeLa cells should represent effects at the mitochondria only. Thus, addition of ang II may lead to a decrease in cellular oxygen consumption, a result that would be consistent with previously described studies; however, this effect appears to be antagonised by both $AT_1$and AT_2 receptors, a

finding which would appear to be biologically inconsistent. Further, as previously discussed, the physiological intracellular concentration of ang II is likely to be in the picomolar range, in accordance with the sub-nanomolar affinity of angiotensin receptors on the cell surface, and the supra-physiological reagent concentrations utilised here may therefore result in non-endogenous effects.

DISCUSSION

D1. Conclusions

The existence of an intracellular RAS has now been extensively investigated and its putative actions have been reported to include mitochondrial dysfunction in the form of increased ROS production, decreased mitochondrial respiratory control and decreased mitochondrial membrane potential (Li et al., 2014, Mariappan et al., 2012, Doughan et al., 2008). RAS inhibition subsequently improves ATP production and overall mitochondrial function (Sumbalova et al., 2010, Taskin et al., 2014). Such effects upon mitochondria may, of course, be mediated indirectly via cell-surface receptors and second messenging systems, or alternate endocrine, paracrine or autocrine actions. Published data had suggested that an intra-mitochondrial RAS might be responsible (Abadir et al., 2011); however, a paucity of data and the fact that many of the studies to date, particularly those reliant upon single antibodies, remain significantly flawed from a methodological point of view, thus raised the question of whether the conclusions from such studies are valid. To explore this issue further, a variety of techniques were utilised in hepatic cells and tissue. Two major hypotheses were proposed at the commencement of the study:

1. ***RAS components can be found within mitochondria (mRAS) derived from rat liver tissue and a liver cell-line, utilizing Western blotting, confocal microscopy and electron microscopy techniques.***

The studies performed for this thesis initially suggested the presence of renin, AT_1R, AT_2R and angiotensin within sub-fractionated mitochondrial compartments in both rat liver tissue and HepG2 cells on Western blotting; renin, ang and ACE co-localising with mitochondria in HepG2 cells on immunofluorescence and confocal microscopy; and ACE, ang, AT_1R and AT_2R in association with mitochondria of rat liver and the HepG2 cell line on immunogold labeling and electron microscopy. However, findings were inconsistent between techniques and tissue/cell-lines, in keeping with a recent American study (Benicky et al., 2012) which reported that six commercially-available antibodies against AT_1R (including the sc-1173 AT_1R antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) utilised for this thesis) lack specificity. The authors demonstrated that each AT_1R antibody (utilised for Western blotting and immunocytochemistry) yielded multiple prominent immunoreactive bands and that the immunostaining patterns were different for each antibody tested. Further, although each antibody did identify a 43 kDa band (target size of AT_1R), the same bands were seen in

AT_1R knockout mice and rat hypothalamic 4B cells not expressing AT_1 receptors. The authors subsequently concluded that none of the tested antibodies met sufficient criteria to be considered specific and suggested that radioligand binding might be the only reliable approach for the assessment of AT_1R physiology.

Initial Western blots also identified a previously unexpected but prominent 55 kDa band, when immunostaining a crude mitochondrial fraction with ACE C-terminal antibody. Thus, the original experimental plan was altered to attempt analysis of the prominent band and in particular to assess whether it represented a novel, mitochondrial-specific form of ACE. However, multiple subsequent fruitless attempts to identify an underlying protein demonstrated that these Western blot findings most likely represent non-specific antibody binding only. The majority of studies investigating the presence of an intra-mitochondrial RAS have relied upon the findings of one antibody, and these studies must now be reviewed in the light of these recent findings.

Abadir *et al* (2011) suggested that AT_2Rs are located upon the inner mitochondrial membrane of rat heart and liver, where they co-localise with endogenous ang II to modulate cellular respiration via mitochondrial nitric oxide production, based upon the use of a single antibody. Similarly, Peters *et al* (1996), who identified renin and ACE in intramitochondrial dense bodies of the rat adrenal zona glomerulosa and cortex after nephrectomy and hemodialysis but relied upon single antibodies and provided no assessment of antibody sensitivity. Close inspection of EM images provided by Peters *et al* show a scattered distribution of immunogold labeling around mitochondria, consistent with non-specific binding. Further, the work of Wanka *et al* (2009) identified the presence of mitochondrial renin, again reliant upon the use of a single antibody. Indeed, the only intra-mitochondrial RAS study to date to utilize radio-labeled ligands demonstrated the presence of a truncated prorenin that was imported into mitochondria in adrenal zona glomerulosa cells endogenously (Clausmeyer et al., 1999). However, this study utilised a laboratory-created construct and therefore does not necessarily reflect a phenomenon that occurs endogenously.

Thus, such qualitative techniques (e.g. Western blotting, immunofluorescence and electron microscopy) contain inherent limitations in that they rely upon the limited specificity of antibodies to definitively prove the existence of intra-mitochondrial RAS components.

2. ***Addition of RAS components to liver cells adversely affects a variety of hepatic mitochondrial functions (as assessed via measurement of oxygen consumption and dynamic fluorescence microscopy); whereas, RAS antagonism leads to improvements in hepatic mitochondrial function.***

Functional experimentation performed for this thesis, via addition of ang II to live HepG2 cells, demonstrated a significant decrease in NADH concentration and a significant increase in mitochondrial membrane potential. Conversely, the addition of Losartan (AT_1R antagonist) significantly decreased intracellular NADH and increased intracellular calcium levels. The use of ang II in HeLa cells, however, led to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration, whilst the addition of Losartan led to a significant decrease in intracellular calcium concentration and a significant increase in mitochondrial membrane potential. Exposure to PD123319, a selective, non-peptide AT_2 receptor antagonist led to a significant decrease in mitochondrial membrane potential, intracellular calcium concentration and NADH concentration. The clear inconsistencies within this data suggest that such functional experimentation, particularly when used at such significantly supra-physiological levels (1 μM – 1 mM), is incapable of separating effects secondary to RAS receptor-ligand binding at the cell-surface, intracellularly or, specifically, intra-mitochondrially. Such data therefore suggests that the effect of ang II on liver mitochondria is unlikely to be direct, and that any positive associations discovered to date are likely to be secondary to methodological flaws. Such findings do not exclude ang II effects upon mitochondrial function, but suggest that any action is likely to occur via one of several intracellular pathways, such as Ca^{2+} signaling via the AT_1R/G-protein/IP_3 system, regulation of gene expression or mitochondrial biogenesis pathways.

This thesis contains unbiased analyses of all known classical RAS components, as well as a targeted evaluation of the presence of ACE via mass spectrometry. The results, as presented here, provide no evidence for the idea of a complete, functioning hepatic mitochondrial RAS of the type postulated by other authors. It remains possible, if unlikely, however, that RAS components may be imported into mitochondria for a specific function in certain tissues under certain specific conditions. Perhaps more likely is that the observed effects of ang II on mitochondrial function (in certain tissues) occurs via its well-established and

ubiquitous intracrine actions upon the nucleus (Erdmann et al., 1996, Huang et al., 2003, Robertson and Khairallah, 1971b), albeit that the specific mechanisms and consequences of such actions require further elucidation (Re and Cook, 2010). Another possibility remains the theory of endosomal escape, whereby the ligand (in this case ang II) leaks from endosomes following receptor-ligand internalisation, becoming free within the cytoplasm to act upon organelles, including mitochondria, and leading to intracrine effects (Cho et al., 2003), a mechanism that forms the basis of many mammalian DNA transfection strategies. Angiotensin receptor (AT_1R or AT_2R) antagonists may therefore protect from such intracrine or mitochondrial ang II action by preventing receptor-ligand endocytosis, and subsequent endosomal escape, rather than via local receptor blockade of putative mitochondrial or other such functional intracellular receptors. Furthermore, there is no evidence to prove the existence of intra-mitochondrial ang II production, a necessary requirement for an intrinsic mitochondrial RAS.

Taken together, therefore, these data exclude the presence of a complete, functioning RAS within hepatic mitochondria, as well as the direct action of intracrine ang II upon mitochondria. If an intrinsic, functional hepatic mitochondrial RAS were to exist, its regulation, roles and actions remain unknown, and the role of ACE2 and the Mas receptor remain similarly unknown. Challenges exist in the examination of the presence of such an intracrine/intracellular system, particularly given the limitations of immunohistochemical techniques, and following such negative experimentation, as presented here, it must be asked whether further experimentation is indeed required to investigate the presence of a hepatic mitochondrial RAS. Repeating the experiments presented here upon isolated mitochondria would be unlikely to yield novel results, although the further study of both isolated mitochondria and intact cells, in conjunction with both *in vivo* and *in vitro* techniques, might be advantageous. In particular, the development of functional strategies that can distinguish intracellular ang II's effects on cytoplasmic, nuclear or mitochondrial receptors and those from extracellular ang II (via cell surface receptors) may help provide further evidence. To this end, the creation of cell-permeable angiotensin probes would aid in the delineation of the site of action of ang II, as would the generation of transgenic animals expressing intracellular ang II proteins, and strategies, such as high-affinity radio-ligand

binding assays, aimed at identifying those molecules endogenously bound to ang II intracellularly (Redding et al., 2010).

Further functional strategies would include pre-treatment of tissues and cell-lines with ang II or inhibitors, to assess effects upon metabolic rate and the utilisation of SiRNA (small interfering ribonucleic acid), cDNA constructs and tissue from knock-out models to investigate the functional effects of under- and over-expression of various RAS components. Functional assessments could also be extended to include OXPHOS (oxidative phosphorylation) measurements (via a Seahorse plate reader (Seahorse Bioscience, North Billerica, MA, USA)), confocal microscopic assessment of cellular ATP levels (via FRET (Fluorescence Resonance Energy Transfer)-based fluorescent probes targeted to the cytoplasm or mitochondrial matrix) and mitochondrial ROS production (via fluorescent probes DHEt (Dihydroethidium) and mitoSox (Molecular probes, Invitrogen, Carlsbad, CA, USA)), as well as measurement of mitochondrial DNA content (via real-time quantitative polymerase chain reaction).

If such assessment of hepatic tissue provides no definitive evidence for the presence of mitochondrial RAS, the exploration of alternate cell and tissue types could be attempted as differential expression of RAS components in various tissues has already been observed (e.g. human liver and aorta predominantly express AT_1 receptor mRNA, whilst human right atrium contains both AT_1 and AT_2 receptor mRNAs). The presence of such mitochondrial RAS components may be limited to certain tissues and conditions, particularly to the adrenal cortex following nephrectomy (Rong et al., 1994, Peters et al., 1996) and may not be present within hepatocytes at all. Future experimentation could therefore be expanded initially to renal and adrenal tissues, and assessment of novel and alternate peptides of the RAS, including AT_4R, BK_1R, BK_2R, & ACE2, could also take place.

D2. Hepatocellular RAS

If we thus exclude the presence of a complete and functional intra-mitochondrial RAS within hepatocytes, we can clarify, to some extent, a mechanism of action for hepatic RAS. Although all classical RAS components (renin, angiotensinogen, ACE, ang II, AT_1R and AT_2R) have been identified in rat and human liver (Paizis et al., 2002, Paizis et al., 2005), hepatic RAS appears to predominantly act via ang II binding to extracellular AT_1Rs. Hepatocyte ang II exposure leads to a decrease in surface ang II receptors, consistent with swift endocytosis and internalisation of the receptor-ligand complex (a process that could be reliant upon cysteine residues) (Jimenez et al., 1999). Such internalisation leads to inhibition of cyclic adenosine monophosphate (cAMP) production and increases in intracellular inositol 4,5-bisphosphate (IP_2), inositol 1,4,5-trisphosphate (IP_3) generation and calcium concentration, suggesting that these second messenger systems are crucial to the downstream intracellular function of ang II (Jimenez et al., 1999). Such intracellular calcium mobilisation following calcium release from ER stores (Spat and Hunyady, 2004) results in activation of the Krebs cycle and oxidative phosphorylation; although over-stimulation of the system may result in adverse effects including mitochondrial calcium overload, mitochondrial membrane depolarisation and reduced efficiency of ATP production (Mizuno et al., 1988, Fournier et al., 2012, Yogev and Pines, 2011).

Other AT_1R-dependant second messaging systems activated include ROS, MAPK, P13-kinase, PKC and NF-κB signal pathways (Zhao et al., 2013, Karimian et al., 2012), all of which may have effects upon mitochondrial physiology or pathophysiology (Hunyady and Catt, 2006, Re et al., 1981), as well as resulting in induction of C-reactive protein (CRP) and its related signal pathway expression (*in vitro* and *in vivo*), ROS production (Zhao et al., 2013) and protection against bile-salt induced apoptosis, via inhibition of ER stress (Karimian et al., 2012).

The addition of exogenous ang II also increases hepatocellular collagen expression, via an AT_1R-NADPH oxidase-mediated pathway (Bataller et al., 2003c), contributing to hepatic fibrosis (Bataller et al., 2000, Hirose et al., 2007, Kim et al., 2008, Ibanez et al., 2007, Ueki et al., 2006, Yang et al., 2005, Tuncer et al., 2003, Bataller et al., 2005, Colmenero et al., 2009, Debernardi-Venon et al., 2007, Sookoian et al., 2005, Terui et al., 2002, Yoshiji et al.,

2005, Yoshiji et al., 2006, Yokohama et al., 2006) and has pro-mitotic and pro–proliferative effects, possibly via an autocrine/paracrine AT_1R-PDGF pathway (Cook et al., 2001). Antagonism of hepatic ACE or AT_1R, however, enhances NRF-1 and PGCV-1 mRNA, resulting in attenuation of age-related mitochondrial DNA decrease, improved mitochondrial function and lower oxidant production (de Cavanagh et al., 2008b).

REFERENCES

(2010) Human Mitochondrial Protein Database.

ABADIR, P. M., FOSTER, D. B., CROW, M., COOKE, C. A., RUCKER, J. J., JAIN, A., SMITH, B. J., BURKS, T. N., COHN, R. D., FEDARKO, N. S., CAREY, R. M., O'ROURKE, B. & WALSTON, J. D. (2011) Identification and characterization of a functional mitochondrial angiotensin system. *Proc Natl Acad Sci U S A,* 108, 14849-54.

ADVANI, A., KELLY, D. J., COX, A. J., WHITE, K. E., ADVANI, S. L., THAI, K., CONNELLY, K. A., YUEN, D., TROGADIS, J., HERZENBERG, A. M., KULISZEWSKI, M. A., LEONG-POI, H. & GILBERT, R. E. (2009) The (Pro)renin receptor: site-specific and functional linkage to the vacuolar H+-ATPase in the kidney. *Hypertension,* 54, 261-9.

AHMED, A. U. & FISHER, P. R. (2009) Import of nuclear-encoded mitochondrial proteins: a cotranslational perspective. *Int Rev Cell Mol Biol,* 273, 49-68.

ALMEIDA, A. P., FRABREGAS, B. C., MADUREIRA, M. M., SANTOS, R. J., CAMPAGNOLE-SANTOS, M. J. & SANTOS, R. A. (2000) Angiotensin-(1-7) potentiates the coronary vasodilatatory effect of bradykinin in the isolated rat heart. *Braz J Med Biol Res,* 33, 709-13.

ANBORGH, P. H., SEACHRIST, J. L., DALE, L. B. & FERGUSON, S. S. (2000) Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors. *Mol Endocrinol,* 14, 2040-53.

BARTON, B. E. (2001) IL-6-like cytokines and cancer cachexia: consequences of chronic inflammation. *Immunol Res,* 23, 41-58.

BASSO, N., CINI, R., PIETRELLI, A., FERDER, L., TERRAGNO, N. A. & INSERRA, F. (2007) Protective effect of long-term angiotensin II inhibition. *Am J Physiol Heart Circ Physiol,* 293, H1351-8.

BASTIANUTTO, C., CLEMENTI, E., CODAZZI, F., PODINI, P., DE GIORGI, F., RIZZUTO, R., MELDOLESI, J. & POZZAN, T. (1995) Overexpression of calreticulin increases the Ca2+ capacity of rapidly exchanging Ca2+ stores and reveals aspects of their lumenal microenvironment and function. *J Cell Biol,* 130, 847-55.

BATALLER, R. & BRENNER, D. A. (2005) Liver fibrosis. *J Clin Invest,* 115, 209-18.

BATALLER, R., GABELE, E., PARSONS, C. J., MORRIS, T., YANG, L., SCHOONHOVEN, R., BRENNER, D. A. & RIPPE, R. A. (2005) Systemic infusion of angiotensin II exacerbates liver fibrosis in bile duct-ligated rats. *Hepatology,* 41, 1046-55.

BATALLER, R., GABELE, E., SCHOONHOVEN, R., MORRIS, T., LEHNERT, M., YANG, L., BRENNER, D. A. & RIPPE, R. A. (2003a) Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver. *Am J Physiol Gastrointest Liver Physiol,* 285, G642-51.

BATALLER, R., GINES, P., NICOLAS, J. M., GORBIG, M. N., GARCIA-RAMALLO, E., GASULL, X., BOSCH, J., ARROYO, V. & RODES, J. (2000) Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology,* 118, 1149-56.

BATALLER, R., SANCHO-BRU, P., GINES, P., LORA, J. M., AL-GARAWI, A., SOLE, M., COLMENERO, J., NICOLAS, J. M., JIMENEZ, W., WEICH, N., GUTIERREZ-RAMOS, J. C., ARROYO, V. & RODES, J. (2003b) Activated human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. *Gastroenterology,* 125, 117-25.

BATALLER, R., SCHWABE, R. F., CHOI, Y. H., YANG, L., PAIK, Y. H., LINDQUIST, J., QIAN, T., SCHOONHOVEN, R., HAGEDORN, C. H., LEMASTERS, J. J. & BRENNER, D. A. (2003c) NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest,* 112, 1383-94.

BEARD, K. M., LU, H., HO, K. & FANTUS, I. G. (2006) Bradykinin augments insulin-stimulated glucose transport in rat adipocytes via endothelial nitric oxide synthase-mediated inhibition of Jun NH2-terminal kinase. *Diabetes,* 55, 2678-87.

BENICKY, J., HAFKO, R., SANCHEZ-LEMUS, E., AGUILERA, G. & SAAVEDRA, J. M. (2012) Six commercially available angiotensin II AT1 receptor antibodies are non-specific. *Cell Mol Neurobiol,* 32, 1353-65.

BENIGNI, A., CORNA, D., ZOJA, C., SONZOGNI, A., LATINI, R., SALIO, M., CONTI, S., ROTTOLI, D., LONGARETTI, L., CASSIS, P., MORIGI, M., COFFMAN, T. M. & REMUZZI, G. (2009) Disruption of the Ang II type 1 receptor promotes longevity in mice. *J Clin Invest,* 119, 524-30.

BHATHAL, P. S. & GROSSMAN, H. J. (1985) Reduction of the increased portal vascular resistance of the isolated perfused cirrhotic rat liver by vasodilators. *J Hepatol,* 1, 325-37.

BKAILY, G., SLEIMAN, S., STEPHAN, J., ASSELIN, C., CHOUFANI, S., KAMAL, M., JACQUES, D., GOBEIL, F., JR. & D'ORLEANS-JUSTE, P. (2003) Angiotensin II AT1 receptor internalization, translocation and de novo synthesis modulate cytosolic and nuclear calcium in human vascular smooth muscle cells. *Can J Physiol Pharmacol,* 81, 274-87.

BOSNYAK, S., JONES, E. S., CHRISTOPOULOS, A., AGUILAR, M. I., THOMAS, W. G. & WIDDOP, R. E. (2011) Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors. *Clin Sci (Lond),* 121, 297-303.

BOTTINGER, L., GUIARD, B., OELJEKLAUS, S., KULAWIAK, B., ZUFALL, N., WIEDEMANN, N., WARSCHEID, B., VAN DER LAAN, M. & BECKER, T. (2013) A complex of Cox4 and mitochondrial Hsp70 plays an important role in the assembly of the cytochrome c oxidase. *Mol Biol Cell*, 24, 609-19.

BRECHLER, V., JONES, P. W., LEVENS, N. R., DE GASPARO, M. & BOTTARI, S. P. (1993) Agonistic and antagonistic properties of angiotensin analogs at the AT2 receptor in PC12W cells. *Regul Pept,* 44, 207-13.

BRINK, M., PRICE, S. R., CHRAST, J., BAILEY, J. L., ANWAR, A., MITCH, W. E. & DELAFONTAINE, P. (2001) Angiotensin II induces skeletal muscle wasting through enhanced protein degradation and down-regulates autocrine insulin-like growth factor I. *Endocrinology,* 142, 1489-96.

BRINK, M., WELLEN, J. & DELAFONTAINE, P. (1996) Angiotensin II causes weight loss and decreases circulating insulin-like growth factor I in rats through a pressor-independent mechanism. *J Clin Invest,* 97, 2509-16.

BROWN, D., BRETON, S., AUSIELLO, D. A. & MARSHANSKY, V. (2009) Sensing, signaling and sorting events in kidney epithelial cell physiology. *Traffic,* 10, 275-84.

BROWN, G. P. & DOUGLAS, J. G. (1982) Angiotensin II binding sites on isolated rat renal brush border membranes. *Endocrinology,* 111, 1830-6.

BROWN, N. J., BLAIS, C., JR., GANDHI, S. K. & ADAM, A. (1998) ACE insertion/deletion genotype affects bradykinin metabolism. *J Cardiovasc Pharmacol,* 32, 373-7.

BRULL, D., DHAMRAIT, S., MYERSON, S., ERDMANN, J., WOODS, D., WORLD, M., PENNELL, D., HUMPHRIES, S., REGITZ-ZAGROSEK, V. & MONTGOMERY, H. (2001) Bradykinin B2BKR receptor polymorphism and left-ventricular growth response. *Lancet,* 358, 1155-6.

BUCHWALOW, I., SAMOILOVA, V., BOECKER, W. & TIEMANN, M. (2011) Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. *Sci Rep,* 1, 28.

CALDWELL, P. R., SEEGAL, B. C., HSU, K. C., DAS, M. & SOFFER, R. L. (1976) Angiotensin-converting enzyme: vascular endothelial localization. *Science,* 191, 1050-1.

CAMARGO DE ANDRADE, M. C., DI MARCO, G. S., DE PAULO CASTRO TEIXEIRA, V., MORTARA, R. A., SABATINI, R. A., PESQUERO, J. B., BOIM, M. A., CARMONA, A. K., SCHOR, N. & CASARINI, D. E. (2006) Expression and localization of N-domain ANG I-converting enzymes in mesangial cells in culture from spontaneously hypertensive rats. *Am J Physiol Renal Physiol,* 290, F364-75.

CAMPBELL, D. J. (1987) Circulating and tissue angiotensin systems. *J Clin Invest,* 79, 1-6.

CAMPBELL, D. J. (1996) Endogenous angiotensin II levels and the mechanism of action of angiotensin-converting enzyme inhibitors and angiotensin receptor type 1 antagonists. *Clin Exp Pharmacol Physiol,* 23 Suppl 3, S125-31.

CAMPBELL, D. J. & HABENER, J. F. (1986) Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. *J Clin Invest,* 78, 31-9.

CAMPBELL, D. J., KLADIS, A. & DUNCAN, A. M. (1994) Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension,* 23, 439-49.

CAMPBELL, D. J., SERNIA, C., THOMAS, W. G. & OLDFIELD, B. J. (1991) Immunccytochemical localization of angiotensinogen in rat brain: dependence of neuronal immunoreactivity on method of tissue processing. *J Neuroendocrinol,* 3, 653-60.

CAREY, R. M. & SIRAGY, H. M. (2003) Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev,* 24, 261-71.

CASSIS, L., HELTON, M., ENGLISH, V. & BURKE, G. (2002) Angiotensin II regulates oxygen consumption. *Am J Physiol Regul Integr Comp Physiol,* 282, R445-53.

CASSIS, P., CONTI, S., REMUZZI, G. & BENIGNI, A. (2010) Angiotensin receptors as determinants of life span. *Pflugers Arch,* 459, 325-32.

CERVENKA, L., VANECKOVA, I., HUSKOVA, Z., VANOURKOVA, Z., ERBANOVA, M., THUMOVA, M., SKAROUPKOVA, P., OPOCENSKY, M., MALY, J., CHABOVA, V. C., TESAR, V., BURGELOVA, M., VIKLICKY, O., TEPLAN, V., ZELIZKO, M., KRAMER, H. J. & NAVAR, L. G. (2008) Pivotal role of angiotensin II receptor subtype 1A in the development of two-kidney, one-clip hypertension: study in angiotensin II receptor subtype 1A knockout mice. *J Hypertens,* 26, 1379-89.

CHAI, S. Y., FERNANDO, R., PECK, G., YE, S. Y., MENDELSOHN, F. A., JENKINS, T. A. & ALBISTON, A. L. (2004) The angiotensin IV/AT4 receptor. *Cell Mol Life Sci,* 61, 2728-37.

CHAI, S. Y., MENDELSOHN, F. A. & PAXINOS, G. (1987) Angiotensin converting enzyme in rat brain visualized by quantitative in vitro autoradiography. *Neuroscience,* 20, 615-27.

CHATTOPADHYAY, S., KARAN, G., SEN, I. & SEN, G. C. (2008) A small region in the angiotensin-converting enzyme distal ectodomain is required for cleavage-secretion of the protein at the plasma membrane. *Biochemistry,* 47, 8335-41.

CHEN, G., LIN, L. X., ZHUANG, W. T., YAO, J., HUANG, H. B., LIANG, J. X., ZHANG, F. L., WEN, J. P., LI, L. T., LIN, M. & LIN, Q. M. (2004) [Effects of captopril on myocardial tissue energy metabolism and inflammation in rats with diabetic cardiomyopathy]. *Di Yi Jun Yi Da Xue Xue Bao,* 24, 827-8, 831.

CHEN, R., MUKHIN, Y. V., GARNOVSKAYA, M. N., THIELEN, T. E., IIJIMA, Y., HUANG, C., RAYMOND, J. R., ULLIAN, M. E. & PAUL, R. V. (2000) A functional angiotensin II receptor-GFP fusion protein: evidence for agonist-dependent nuclear translocation. *Am J Physiol Renal Physiol,* 279, F440-8.

CHO, Y. W., KIM, J. D. & PARK, K. (2003) Polycation gene delivery systems: escape from endosomes to cytosol. *J Pharm Pharmacol,* 55, 721-34.

CLARK, A. F., SHARP, M. G., MORLEY, S. D., FLEMING, S., PETERS, J. & MULLINS, J. J. (1997) Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology. *J Biol Chem,* 272, 18185-90.

CLAUSMEYER, S., REINECKE, A., FARRENKOPF, R., UNGER, T. & PETERS, J. (2000) Tissue-specific expression of a rat renin transcript lacking the coding sequence for the prefragment and its stimulation by myocardial infarction. *Endocrinology,* 141, 2963-70.

CLAUSMEYER, S., STURZEBECHER, R. & PETERS, J. (1999) An alternative transcript of the rat renin gene can result in a truncated prorenin that is transported into adrenal mitochondria. *Circ Res,* 84, 337-44.

COGAN, M. G. (1990) Angiotensin II: a powerful controller of sodium transport in the early proximal tubule. *Hypertension,* 15, 451-8.

COLMENERO, J., BATALLER, R., SANCHO-BRU, P., DOMINGUEZ, M., MORENO, M., FORNS, X., BRUGUERA, M., ARROYO, V., BRENNER, D. A. & GINES, P. (2009) Effects of losartan on hepatic expression of nonphagocytic NADPH oxidase and fibrogenic genes in patients with chronic hepatitis C. *Am J Physiol Gastrointest Liver Physiol,* 297, G726-34.

COLQUHOUN, E. Q., HETTIARACHCHI, M., YE, J. M., RICHTER, E. A., HNIAT, A. J., RATTIGAN, S. & CLARK, M. G. (1988) Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci,* 43, 1747-54.

COOK, J. L., MILLS, S. J., NAQUIN, R., ALAM, J. & RE, R. N. (2006) Nuclear accumulation of the AT1 receptor in a rat vascular smooth muscle cell line: effects upon signal transduction and cellular proliferation. *J Mol Cell Cardiol,* 40, 696-707.

COOK, J. L., MILLS, S. J., NAQUIN, R. T., ALAM, J. & RE, R. N. (2007) Cleavage of the angiotensin II type 1 receptor and nuclear accumulation of the cytoplasmic carboxy-terminal fragment. *Am J Physiol Cell Physiol,* 292, C1313-22.

COOK, J. L., RE, R., ALAM, J., HART, M. & ZHANG, Z. (2004) Intracellular angiotensin II fusion protein alters AT1 receptor fusion protein distribution and activates CREB. *J Mol Cell Cardiol,* 36, 75-90.

COOK, J. L., ZHANG, Z. & RE, R. N. (2001) In vitro evidence for an intracellular site of angiotensin action. *Circ Res,* 89, 1138-46.

COSTEROUSSE, O., ALLEGRINI, J., LOPEZ, M. & ALHENC-GELAS, F. (1993) Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J,* 290 (Pt 1), 33-40.

CSORDAS, G., RENKEN, C., VARNAI, P., WALTER, L., WEAVER, D., BUTTLE, K. F., BALLA, T., MANNELLA, C. A. & HAJNOCZKY, G. (2006) Structural and functional features and significance of the physical linkage between ER and mitochondria. *J Cell Biol,* 174, 915-21.

CSORDAS, G., VARNAI, P., GOLENAR, T., ROY, S., PURKINS, G., SCHNEIDER, T. G., BALLA, T. & HAJNOCZKY, G. (2010) Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol Cell,* 39, 121-32.

DANSER, A. H. (2009) (Pro)renin receptor and vacuolar H+-ATPase. *Hypertension,* 54, 219-21.

DANSER, A. H., ADMIRAAL, P. J., DERKX, F. H. & SCHALEKAMP, M. A. (1998) Angiotensin I-to-II conversion in the human renal vascular bed. *J Hypertens,* 16, 2051-6.

DANSER, A. H., SCHALEKAMP, M. A., BAX, W. A., VAN DEN BRINK, A. M., SAXENA, P. R., RIEGGER, G. A. & SCHUNKERT, H. (1995) Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation,* 92, 1387-8.

DANSER, A. H., VAN KATS, J. P., ADMIRAAL, P. J., DERKX, F. H., LAMERS, J. M., VERDOUW, P. D., SAXENA, P. R. & SCHALEKAMP, M. A. (1994) Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension,* 24, 37-48.

DARBY, I. A., ALDRED, P., CRAWFORD, R. J., FERNLEY, R. T., NIALL, H. D., PENSCHOW, J. D., RYAN, G. B. & COGHLAN, J. P. (1985) Renin gene expression in vessels of the ovine renal cortex. *J Hypertens,* 3, 9-11.

DARBY, I. A. & SERNIA, C. (1995) In situ hybridization and immunohistochemistry of renal angiotensinogen in neonatal and adult rat kidneys. *Cell Tissue Res,* 281, 197-206.

DE BRITO, O. M. & SCORRANO, L. (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature,* 456, 605-10.

DE CAVANAGH, E. M., FERDER, L., TOBLLI, J. E., PIOTRKOWSKI, B., STELLA, I., FRAGA, C. G. & INSERRA, F. (2008a) Renal mitochondrial impairment is attenuated by AT1 blockade in experimental Type I diabetes. *Am J Physiol Heart Circ Physiol,* 294, H456-65.

DE CAVANAGH, E. M., FERDER, M., INSERRA, F. & FERDER, L. (2009) Angiotensin II, mitochondria, cytoskeletal, and extracellular matrix connections: an integrating viewpoint. *Am J Physiol Heart Circ Physiol,* 296, H550-8.

DE CAVANAGH, E. M., FLORES, I., FERDER, M., INSERRA, F. & FERDER, L. (2008b) Renin-angiotensin system inhibitors protect against age-related changes in rat liver mitochondrial DNA content and gene expression. *Exp Gerontol,* 43, 919-28.

DE CAVANAGH, E. M., INSERRA, F. & FERDER, L. (2011) Angiotensin II blockade: a strategy to slow ageing by protecting mitochondria? *Cardiovasc Res,* 89, 31-40.

DE CAVANAGH, E. M., INSERRA, F., FERDER, M. & FERDER, L. (2007) From mitochondria to disease: role of the renin-angiotensin system. *Am J Nephrol,* 27, 545-53.

DE CAVANAGH, E. M., PIOTRKOWSKI, B., BASSO, N., STELLA, I., INSERRA, F., FERDER, L. & FRAGA, C. G. (2003) Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. *Faseb J,* 17, 1096-8.

DE CAVANAGH, E. M., TOBLLI, J. E., FERDER, L., PIOTRKOWSKI, B., STELLA, I., FRAGA, C. G. & INSERRA, F. (2005) Angiotensin II blockade improves mitochondrial function in spontaneously hypertensive rats. *Cell Mol Biol (Noisy-le-grand),* 51, 573-8.

DE CAVANAGH, E. M., TOBLLI, J. E., FERDER, L., PIOTRKOWSKI, B., STELLA, I. & INSERRA, F. (2006) Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine. *Am J Physiol Regul Integr Comp Physiol,* 290, R1616-25.

DE GASPARO, M., CATT, K. J., INAGAMI, T., WRIGHT, J. W. & UNGER, T. (2000) International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev,* 52, 415-72.

DE MELLO, W. C. (1998) Intracellular angiotensin II regulates the inward calcium current in cardiac myocytes. *Hypertension,* 32, 976-82.

DE MELLO, W. C. (2006) Renin increments the inward calcium current in the failing heart. *J Hypertens,* 24, 1181-6.

DEBERNARDI-VENON, W., MARTINI, S., BIASI, F., VIZIO, B., TERMINE, A., POLI, G., BRUNELLO, F., ALESSANDRIA, C., BONARDI, R., SARACCO, G., RIZZETTO, M.

& MARZANO, A. (2007) AT1 receptor antagonist Candesartan in selected cirrhotic patients: effect on portal pressure and liver fibrosis markers. *J Hepatol,* 46, 1026-33.

DECUYPERE, J. P., MONACO, G., BULTYNCK, G., MISSIAEN, L., DE SMEDT, H. & PARYS, J. B. (2011) The IP(3) receptor-mitochondria connection in apoptosis and autophagy. *Biochim Biophys Acta,* 1813, 1003-13.

DENG, A., TANG, T., SINGH, P., WANG, C., SATRIANO, J., THOMSON, S. C. & BLANTZ, R. C. (2009) Regulation of oxygen utilization by angiotensin II in chronic kidney disease. *Kidney Int,* 75, 197-204.

DESCHEPPER, C. F., MELLON, S. H., CUMIN, F., BAXTER, J. D. & GANONG, W. F. (1986) Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat. *Proc Natl Acad Sci U S A,* 83, 7552-6.

DIKALOVA, A. E., BIKINEYEVA, A. T., BUDZYN, K., NAZAREWICZ, R. R., MCCANN, L., LEWIS, W., HARRISON, D. G. & DIKALOV, S. I. (2010) Therapeutic targeting of mitochondrial superoxide in hypertension. *Circ Res,* 107, 106-16.

DOI, Y., ATARASHI, K., FRANCO-SAENZ, R. & MULROW, P. J. (1984) Effect of changes in sodium or potassium balance, and nephrectomy, on adrenal renin and aldosterone concentrations. *Hypertension,* 6, I124-9.

DONOGHUE, M., HSIEH, F., BARONAS, E., GODBOUT, K., GOSSELIN, M., STAGLIANO, N., DONOVAN, M., WOOLF, B., ROBISON, K., JEYASEELAN, R., BREITBART, R. E. & ACTON, S. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res,* 87, E1-9.

DOUGHAN, A. & DIKALOV, S. (2005) Angiotensin II increases mitochondrial superoxide production in endothelial cells: a potential link between NADPH oxidase and mitochondrial dysfunction. *Free Radic Biol Med(in Davies, K.; SFRBM 12th Annual Meeting: Program and Abstracts),* 39 (Suppl. 1), S23.

DOUGHAN, A. K., HARRISON, D. G. & DIKALOV, S. I. (2008) Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res,* 102, 488-96.

DOUGLAS, J. G. (1987) Angiotensin receptor subtypes of the kidney cortex. *Am J Physiol,* 253, F1-7.

DREHER, D., JORNOT, L. & JUNOD, A. F. (1995) Effects of hypoxanthine-xanthine oxidase on Ca2+ stores and protein synthesis in human endothelial cells. *Circ Res,* 76, 388-95.

DULIN, N. O., ERNSBERGER, P., SUCIU, D. J. & DOUGLAS, J. G. (1994) Rabbit renal epithelial angiotensin II receptors. *Am J Physiol,* 267, F776-82.

DZAU, V. J. (1989) Multiple pathways of angiotensin production in the blood vessel wall: evidence, possibilities and hypotheses. *J Hypertens,* 7, 933-6.

DZAU, V. J., BURT, D. W. & PRATT, R. E. (1988) Molecular biology of the renin-angiotensin system. *Am J Physiol,* 255, F563-73.

EKKER, M., TRONIK, D. & ROUGEON, F. (1989) Extra-renal transcription of the renin genes in multiple tissues of mice and rats. *Proc Natl Acad Sci U S A,* 86, 5155-8.

ERDMANN, B., FUXE, K. & GANTEN, D. (1996) Subcellular localization of angiotensin II immunoreactivity in the rat cerebellar cortex. *Hypertension,* 28, 818-24.

ERMAK, G. & DAVIES, K. J. (2002) Calcium and oxidative stress: from cell signaling to cell death. *Mol Immunol,* 38, 713-21.

FERDER, L., INSERRA, F., ROMANO, L., ERCOLE, L. & PSZENNY, V. (1993) Effects of angiotensin-converting enzyme inhibition on mitochondrial number in the aging mouse. *Am J Physiol,* 265, C15-8.

FERGUSON, S. S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev,* 53, 1-24.

FERNANDES, F. B., PLAVNIK, F. L., TEIXEIRA, A. M., CHRISTOFALO, D. M., AJZEN, S. A., HIGA, E. M., RONCHI, F. A., SESSO, R. C. & CASARINI, D. E. (2008) Association of urinary N-domain Angiotensin I-converting enzyme with plasma inflammatory markers and endothelial function. *Mol Med,* 14, 429-35.

FLORYK, D. & HOUSTEK, J. (1999) Tetramethyl rhodamine methyl ester (TMRM) is suitable for cytofluorometric measurements of mitochondrial membrane potential in cells treated with digitonin. *Biosci Rep,* 19, 27-34.

FOLSCH, H., GUIARD, B., NEUPERT, W. & STUART, R. A. (1996) Internal targeting signal of the BCS1 protein: a novel mechanism of import into mitochondria. *EMBO J,* 15, 479-87.

FOURNIER, D., LUFT, F. C., BADER, M., GANTEN, D. & ANDRADE-NAVARRO, M. A. (2012) Emergence and evolution of the renin-angiotensin-aldosterone system. *J Mol Med (Berl),* 90, 495-508.

FOWLER, J. D., KRUETH, S. B., BERNLOHR, D. A. & KATZ, S. A. (2009) Renin dynamics in adipose tissue: adipose tissue control of local renin concentrations. *Am J Physiol Endocrinol Metab,* 296, E343-50.

FRIEDMAN, J. R., LACKNER, L. L., WEST, M., DIBENEDETTO, J. R., NUNNARI, J. & VOELTZ, G. K. (2011) ER tubules mark sites of mitochondrial division. *Science,* 334, 358-62.

FRIEDMAN, S. L. (2008) Mechanisms of hepatic fibrogenesis. *Gastroenterology,* 134, 1655-69.

FUXE, K., GANTEN, D., HOKFELT, T. & BOLME, P. (1976) Immunohistochemical evidence for the existence of angiotensin II-containing nerve terminals in the brain and spinal cord in the rat. *Neurosci Lett,* 2, 229-34.

FUXE, K., GANTEN, D., HOKFELT, T., LOCATELLI, V., POULSEN, K., STOCK, G., RIX, E. & TAUGNER, R. (1980) Renin-like immunocytochemical activity in the rat and mouse brain. *Neurosci Lett,* 18, 245-50.

FYHRQUIST, F. & SAIJONMAA, O. (2008) Renin-angiotensin system revisited. *J Intern Med,* 264, 224-36.

GANONG, W. F. (1995) Reproduction and the renin-angiotensin system. *Neurosci Biobehav Rev,* 19, 241-50.

GARLID, K. D., JABUREK, M. & JEZEK, P. (1998) The mechanism of proton transport mediated by mitochondrial uncoupling proteins. *FEBS Lett,* 438, 10-4.

GARNIER, A., ZOLL, J., FORTIN, D., N'GUESSAN, B., LEFEBVRE, F., GENY, B., METTAUER, B., VEKSLER, V. & VENTURA-CLAPIER, R. (2009) Control by circulating factors of mitochondrial function and transcription cascade in heart failure: a role for endothelin-1 and angiotensin II. *Circ Heart Fail,* 2, 342-50.

GEE, K. R., BROWN, K. A., CHEN, W. N., BISHOP-STEWART, J., GRAY, D. & JOHNSON, I. (2000) Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium,* 27, 97-106.

GEMBARDT, F., STERNER-KOCK, A., IMBODEN, H., SPALTEHOLZ, M., REIBITZ, F., SCHULTHEISS, H. P., SIEMS, W. E. & WALTHER, T. (2005) Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents. *Peptides,* 26, 1270-7.

GIRONACCI, M. M., VALERA, M. S., YUJNOVSKY, I. & PENA, C. (2004) Angiotensin-(1-7) inhibitory mechanism of norepinephrine release in hypertensive rats. *Hypertension,* 44, 783-7.

GONZALEZ-GAITAN, M. (2003) Signal dispersal and transduction through the endocytic pathway. *Nat Rev Mol Cell Biol,* 4, 213-24.

GRACE, J. A., HERATH, C. B., MAK, K. Y., BURRELL, L. M. & ANGUS, P. W. (2012) Update on new aspects of the renin-angiotensin system in liver disease: clinical implications and new therapeutic options. *Clin Sci (Lond),* 123, 225-39.

GRECO, S., ELIA, M. G., MUSCELLA, A., STORELLI, C. & MARSIGLIANTE, S. (2002) AT1 angiotensin II receptor mediates intracellular calcium mobilization in normal and cancerous breast cells in primary culture. *Cell Calcium,* 32, 1-10.

GROBE, J. L., DER SARKISSIAN, S., STEWART, J. M., MESZAROS, J. G., RAIZADA, M. K. & KATOVICH, M. J. (2007) ACE2 overexpression inhibits hypoxia-induced collagen production by cardiac fibroblasts. *Clin Sci (Lond),* 113, 357-64.

GROVER, A. K., SAMSON, S. E., FOMIN, V. P. & WERSTIUK, E. S. (1995) Effects of peroxide and superoxide on coronary artery: ANG II response and sarcoplasmic reticulum Ca2+ pump. *Am J Physiol,* 269, C546-53.

GWATHMEY, T. M., PENDERGRASS, K. D., REID, S. D., ROSE, J. C., DIZ, D. I. & CHAPPELL, M. C. (2010) Angiotensin-(1-7)-angiotensin-converting enzyme 2 attenuates reactive oxygen species formation to angiotensin II within the cell nucleus. *Hypertension,* 55, 166-71.

GWATHMEY, T. M., SHALTOUT, H. A., PENDERGRASS, K. D., PIRRO, N. T., FIGUEROA, J. P., ROSE, J. C., DIZ, D. I. & CHAPPELL, M. C. (2009) Nuclear angiotensin II type 2 (AT2) receptors are functionally linked to nitric oxide production. *Am J Physiol Renal Physiol,* 296, F1484-93.

HABIBI, J., WHALEY-CONNELL, A., HAYDEN, M. R., DEMARCO, V. G., SCHNEIDER, R., SOWERS, S. D., KARUPARTHI, P., FERRARIO, C. M. & SOWERS, J.

R. (2008) Renin inhibition attenuates insulin resistance, oxidative stress, and pancreatic remodeling in the transgenic Ren2 rat. *Endocrinology,* 149, 5643-53.

HABOUZIT, E., RICHARD, H., SANCHEZ, H., KOULMANN, N., SERRURIER, B., MONNET, R., VENTURA-CLAPIER, R. & BIGARD, X. (2009) Decreased muscle ACE activity enhances functional response to endurance training in rats, without change in muscle oxidative capacity or contractile phenotype. *J Appl Physiol,* 107, 346-53.

HACKENTHAL, E., PAUL, M., GANTEN, D. & TAUGNER, R. (1990) Morphology, physiology, and molecular biology of renin secretion. *Physiol Rev,* 70, 1067-116.

HAGEN, TM., YOWE DL., BARTHOLOMEW, JC., WEHR, CM., DO, KL., PARK, J-Y. & BRUCE N. AMES, BN. (1997) Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci U S A*, 94, 3064–3069.

HAJNOCZKY, G., CSORDAS, G. & YI, M. (2002) Old players in a new role: mitochondria-associated membranes, VDAC, and ryanodine receptors as contributors to calcium signal propagation from endoplasmic reticulum to the mitochondria. *Cell Calcium,* 32, 363-77.

HALLER, H., LINDSCHAU, C., ERDMANN, B., QUASS, P. & LUFT, F. C. (1996) Effects of intracellular angiotensin II in vascular smooth muscle cells. *Circ Res,* 79, 765-72.

HALLER, H., LINDSCHAU, C., QUASS, P. & LUFT, F. C. (1999) Intracellular actions of angiotensin II in vascular smooth muscle cells. *J Am Soc Nephrol,* 10 Suppl 11, S75-83.

HARRIS, P. J. & NAVAR, L. G. (1985) Tubular transport responses to angiotensin. *Am J Physiol,* 248, F621-30.

HAYASHI, T. & SU, T. P. (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell,* 131, 596-610.

HAYNES, C. M. & RON, D. (2010) The mitochondrial UPR - protecting organelle protein homeostasis. *J Cell Sci,* 123, 3849-55.

HEIN, L., BARSH, G. S., PRATT, R. E., DZAU, V. J. & KOBILKA, B. K. (1995) Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature,* 377, 744-7.

HEIN, L., MEINEL, L., PRATT, R. E., DZAU, V. J. & KOBILKA, B. K. (1997) Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol,* 11, 1266-77.

HERATH, C. B., LUBEL, J. S., JIA, Z., VELKOSKA, E., CASLEY, D., BROWN, L., TIKELLIS, C., BURRELL, L. M. & ANGUS, P. W. (2009) Portal pressure responses and angiotensin peptide production in rat liver are determined by relative activity of ACE and ACE2. *Am J Physiol Gastrointest Liver Physiol,* 297, G98-G106.

HILLENKAMP, F., KARAS, M., BEAVIS, R. C. & CHAIT, B. T. (1991) Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal Chem,* 63, 1193A-1203A.

HIROSE, A., ONO, M., SAIBARA, T., NOZAKI, Y., MASUDA, K., YOSHIOKA, A., TAKAHASHI, M., AKISAWA, N., IWASAKI, S., OBEN, J. A. & ONISHI, S. (2007) Angiotensin II type 1 receptor blocker inhibits fibrosis in rat nonalcoholic steatohepatitis. *Hepatology,* 45, 1375-81.

HITOMI, H., KIYOMOTO, H. & NISHIYAMA, A. (2007) Angiotensin II and oxidative stress. *Curr Opin Cardiol,* 22, 311-5.

HOBART, P. M., FOGLIANO, M., O'CONNOR, B. A., SCHAEFER, I. M. & CHIRGWIN, J. M. (1984) Human renin gene: structure and sequence analysis. *Proc Natl Acad Sci U S A,* 81, 5026-30.

HOLLENBERG, N. K., CHENITZ, W. R., ADAMS, D. F. & WILLIAMS, G. H. (1974) Reciprocal influence of salt intake on adrenal glomerulosa and renal vascular responses to angiotensin II in normal man. *J Clin Invest,* 54, 34-42.

HOUILLIER, P., CHAMBREY, R., ACHARD, J. M., FROISSART, M., POGGIOLI, J. & PAILLARD, M. (1996) Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. *Kidney Int,* 50, 1496-505.

HUANG, J., HARA, Y., ANRATHER, J., SPETH, R. C., IADECOLA, C. & PICKEL, V. M. (2003) Angiotensin II subtype 1A (AT1A) receptors in the rat sensory vagal complex: subcellular localization and association with endogenous angiotensin. *Neuroscience,* 122, 21-36.

HUNDAL, T., NORLING, B. & ERNSTER, L. (1984) The oligomycin sensitivity conferring protein (OSCP) of beef heart mitochondria: studies of its binding to F1 and its function. *J Bioenerg Biomembr,* 16, 535-50.

HUNYADY, L. (1999) Molecular mechanisms of angiotensin II receptor internalization. *J Am Soc Nephrol,* 10 Suppl 11, S47-56.

HUNYADY, L. & CATT, K. J. (2006) Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol,* 20, 953-70.

HUNYADY, L., CATT, K. J., CLARK, A. J. & GABORIK, Z. (2000) Mechanisms and functions of AT(1) angiotensin receptor internalization. *Regul Pept,* 91, 29-44.

IBANEZ, P., SOLIS, N., PIZARRO, M., AGUAYO, G., DUARTE, I., MIQUEL, J. F., ACCATINO, L. & ARRESE, M. (2007) Effect of losartan on early liver fibrosis development in a rat model of nonalcoholic steatohepatitis. *J Gastroenterol Hepatol,* 22, 846-51.

IMAI, T., MIYAZAKI, H., HIROSE, S., HORI, H., HAYASHI, T., KAGEYAMA, R., OHKUBO, H., NAKANISHI, S. & MURAKAMI, K. (1983) Cloning and sequence analysis of cDNA for human renin precursor. *Proc Natl Acad Sci U S A,* 80, 7405-9.

IMAIZUMI, T., TAKESHITA, A., NAKAMURA, N., SAKAI, K., HIROOKA, Y., SUZUKI, S., YOSHIDA, M. & NAKAMURA, M. (1990) Effects of captopril on forearm oxygen consumption during dynamic handgrip exercise in patients with congestive heart failure. *Jpn Heart J,* 31, 817-28.

IMIG, J. D., NAVAR, G. L., ZOU, L. X., O'REILLY, K. C., ALLEN, P. L., KAYSEN, J. H., HAMMOND, T. G. & NAVAR, L. G. (1999) Renal endosomes contain angiotensin peptides, converting enzyme, and AT(1A) receptors. *Am J Physiol,* 277, F303-11.

INGELFINGER, J. R., JUNG, F., DIAMANT, D., HAVERAN, L., LEE, E., BREM, A. & TANG, S. S. (1999) Rat proximal tubule cell line transformed with origin-defective SV40 DNA: autocrine ANG II feedback. *Am J Physiol,* 276, F218-27.

INGERT, C., GRIMA, M., COQUARD, C., BARTHELMEBS, M. & IMBS, J. L. (2002) Contribution of angiotensin II internalization to intrarenal angiotensin II levels in rats. *Am J Physiol Renal Physiol,* 283, F1003-10.

IVANKOVIC, M., CUKUSIC, A., GOTIC, I., SKROBOT, N., MATIJASIC, M., POLANCEC, D. & RUBELJ, I. (2007) Telomerase activity in HeLa cervical carcinoma cell line proliferation. *Biogerontology,* 8, 163-72.

IWANAMI, J., MOGI, M., IWAI, M. & HORIUCHI, M. (2009) Inhibition of the renin-angiotensin system and target organ protection. *Hypertens Res,* 32, 229-37.

JABUREK, M., VARECHA, M., GIMENO, R. E., DEMBSKI, M., JEZEK, P., ZHANG, M., BURN, P., TARTAGLIA, L. A. & GARLID, K. D. (1999) Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J Biol Chem,* 274, 26003-7.

JIMENEZ, E., CARO, M. C., MARSIGLIANTE, S. & MONTIEL, M. (1999) Angiotensin II receptor internalization and signaling in isolated rat hepatocytes. *Biochem Pharmacol,* 57, 1125-31.

JONDEAU, G., DIB, J. C., DUBOURG, O. & BOURDARIAS, J. P. (1997) Relation of functional improvement in congestive heart failure after quinapril therapy to peripheral limitation. *Am J Cardiol,* 79, 635-8.

KANEDA, K., EKATAKSIN, W., SOGAWA, M., MATSUMURA, A., CHO, A. & KAWADA, N. (1998) Endothelin-1-induced vasoconstriction causes a significant increase in portal pressure of rat liver: localized constrictive effect on the distal segment of preterminal portal venules as revealed by light and electron microscopy and serial reconstruction. *Hepatology,* 27, 735-47.

KARIMIAN, G., BUIST-HOMAN, M., MIKUS, B., HENNING, R. H., FABER, K. N. & MOSHAGE, H. (2012) Angiotensin II protects primary rat hepatocytes against bile salt-induced apoptosis. *PLoS One,* 7, e52647.

KAWAS, L. H., MCCOY, A. T., YAMAMOTO, B. J., WRIGHT, J. W. & HARDING, J. W. (2012) Development of angiotensin IV analogs as hepatocyte growth factor/Met modifiers. *J Pharmacol Exp Ther,* 340, 539-48.

KEM, D. C. & BROWN, R. D. (1990) Renin--from beginning to end. *N Engl J Med,* 323, 1136-7.

KERINS, D. M., HAO, Q. & VAUGHAN, D. E. (1995) Angiotensin induction of PAI-1 expression in endothelial cells is mediated by the hexapeptide angiotensin IV. *J Clin Invest,* 96, 2515-20.

KIM, M. Y., BAIK, S. K., PARK, D. H., JANG, Y. O., SUK, K. T., YEA, C. J., LEE, I. Y., KIM, J. W., KIM, H. S., KWON, S. O., CHO, M. Y., KO, S. B., CHANG, S. J., UM, S. H. & HAN, K. H. (2008) Angiotensin receptor blockers are superior to angiotensin-converting enzyme inhibitors in the suppression of hepatic fibrosis in a bile duct-ligated rat model. *J Gastroenterol,* 43, 889-96.

KIMURA, S., ZHANG, G. X., NISHIYAMA, A., SHOKOJI, T., YAO, L., FAN, Y. Y., RAHMAN, M. & ABE, Y. (2005) Mitochondria-derived reactive oxygen species and vascular MAP kinases: comparison of angiotensin II and diazoxide. *Hypertension,* 45, 438-44.

KISHI, K., MUROMOTO, N., NAKAYA, Y., MIYATA, I., HAGI, A., HAYASHI, H. & EBINA, Y. (1998) Bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway. *Diabetes,* 47, 550-8.

KOBORI, H., HARRISON-BERNARD, L. M. & NAVAR, L. G. (2001a) Enhancement of angiotensinogen expression in angiotensin II-dependent hypertension. *Hypertension,* 37, 1329-35.

KOBORI, H., HARRISON-BERNARD, L. M. & NAVAR, L. G. (2001b) Expression of angiotensinogen mRNA and protein in angiotensin II-dependent hypertension. *J Am Soc Nephrol,* 12, 431-9.

KOBORI, H., HARRISON-BERNARD, L. M. & NAVAR, L. G. (2002) Urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production. *Kidney Int,* 61, 579-85.

KOBORI, H., OZAWA, Y., SUZAKI, Y., PRIETO-CARRASQUERO, M. C., NISHIYAMA, A., SHOJI, T., COHEN, E. P. & NAVAR, L. G. (2006) Young Scholars Award Lecture: Intratubular angiotensinogen in hypertension and kidney diseases. *Am J Hypertens,* 19, 541-50.

KUMAR, R., SINGH, V. P. & BAKER, K. M. (2007) The intracellular renin-angiotensin system: a new paradigm. *Trends Endocrinol Metab,* 18, 208-14.

KUZNETSOV, A. V., VEKSLER, V., GELLERICH, F. N., SAKS, V., MARGREITER, R. & KUNZ, W. S. (2008) Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc,* 3, 965-76.

LAMBERT, D. W., YARSKI, M., WARNER, F. J., THORNHILL, P., PARKIN, E. T., SMITH, A. I., HOOPER, N. M. & TURNER, A. J. (2005) Tumor necrosis factor-alpha

convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem,* 280, 30113-9.

LANTELME, P., ROHRWASSER, A., GOCIMAN, B., HILLAS, E., CHENG, T., PETTY, G., THOMAS, J., XIAO, S., ISHIGAMI, T., HERRMANN, T., TERREROS, D. A., WARD, K. & LALOUEL, J. M. (2002) Effects of dietary sodium and genetic background on angiotensinogen and Renin in mouse. *Hypertension,* 39, 1007-14.

LARKIN, J. E., FRANK, B. C., GASPARD, R. M., DUKA, I., GAVRAS, H. & QUACKENBUSH, J. (2004) Cardiac transcriptional response to acute and chronic angiotensin II treatments. *Physiol Genomics,* 18, 152-66.

LATT, S. A. & STETTEN, G. (1976) Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J Histochem Cytochem,* 24, 24-33.

LATT, S. A., STETTEN, G., JUERGENS, L. A., WILLARD, H. F. & SCHER, C. D. (1975) Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. *J Histochem Cytochem,* 23, 493-505.

LAU, T., CARLSSON, P. O. & LEUNG, P. S. (2004) Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets. *Diabetologia,* 47, 240-8.

LAVRENTYEV, E. N., ESTES, A. M. & MALIK, K. U. (2007) Mechanism of high glucose induced angiotensin II production in rat vascular smooth muscle cells. *Circ Res,* 101, 455-64.

LAVRENTYEV, E. N. & MALIK, K. U. (2009) High glucose-induced Nox1-derived superoxides downregulate PKC-betaII, which subsequently decreases ACE2 expression and ANG(1-7) formation in rat VSMCs. *Am J Physiol Heart Circ Physiol,* 296, H106-18.

LEARY, S. C., MICHAUD, D., LYONS, C. N., HALE, T. M., BUSHFIELD, T. L., ADAMS, M. A. & MOYES, C. D. (2002) Bioenergetic remodeling of heart during treatment of spontaneously hypertensive rats with enalapril. *Am J Physiol Heart Circ Physiol,* 283, H540-8.

LEE, D. K., LANCA, A. J., CHENG, R., NGUYEN, T., JI, X. D., GOBEIL, F., JR., CHEMTOB, S., GEORGE, S. R. & O'DOWD, B. F. (2004) Agonist-independent nuclear

localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors. *J Biol Chem,* 279, 7901-8.

LEE, M. Y. & GRIENDLING, K. K. (2008) Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal,* 10, 1045-59.

LEE-KIRSCH, M. A., GAUDET, F., CARDOSO, M. C. & LINDPAINTNER, K. (1999) Distinct renin isoforms generated by tissue-specific transcription initiation and alternative splicing. *Circ Res,* 84, 240-6.

LEEB-LUNDBERG, L. M., MARCEAU, F., MULLER-ESTERL, W., PETTIBONE, D. J. & ZURAW, B. L. (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev,* 57, 27-77.

LENKEI, Z., PALKOVITS, M., CORVOL, P. & LLORENS-CORTES, C. (1997) Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. *Front Neuroendocrinol,* 18, 383-439.

LEUNG, P. S. (2007) The physiology of a local renin-angiotensin system in the pancreas. *J Physiol,* 580, 31-7.

LEUNG, P. S. & CARLSSON, P. O. (2001) Tissue renin-angiotensin system: its expression, localization, regulation and potential role in the pancreas. *J Mol Endocrinol,* 26, 155-64.

LEUNG, P. S., CHAN, H. C., FU, L. X. & WONG, P. Y. (1997) Localization of angiotensin II receptor subtypes AT1 and AT2 in the pancreas of rodents. *J Endocrinol,* 153, 269-74.

LEUNG, P. S., CHAN, W. P., WONG, T. P. & SERNIA, C. (1999) Expression and localization of the renin-angiotensin system in the rat pancreas. *J Endocrinol,* 160, 13-9.

LEUNG, P. S. & IP, S. P. (2006) Pancreatic acinar cell: its role in acute pancreatitis. *Int J Biochem Cell Biol,* 38, 1024-30.

LEUNG, P. S. & SERNIA, C. (2003) The renin-angiotensin system and male reproduction: new functions for old hormones. *J Mol Endocrinol,* 30, 263-70.

LI, G., WANG, M., HAO, L., LOO, W. T., JIN, L., CHEUNG, M. N., CHOW, L. W. & NG, E. L. (2014) Angiotensin II induces mitochondrial dysfunction and promotes apoptosis

via JNK signalling pathway in primary mouse calvaria osteoblast. *Arch Oral Biol,* 59, 513-523.

LI, W., MOORE, M. J., VASILIEVA, N., SUI, J., WONG, S. K., BERNE, M. A., SOMASUNDARAN, M., SULLIVAN, J. L., LUZURIAGA, K., GREENOUGH, T. C., CHOE, H. & FARZAN, M. (2003) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature,* 426, 450-4.

LI, X. C., CARRETERO, O. A., NAVAR, L. G. & ZHUO, J. L. (2006) AT1 receptor-mediated accumulation of extracellular angiotensin II in proximal tubule cells: role of cytoskeleton microtubules and tyrosine phosphatases. *Am J Physiol Renal Physiol,* 291, F375-83.

LI, X. C., HOPFER, U. & ZHUO, J. L. (2009) AT1 receptor-mediated uptake of angiotensin II and NHE-3 expression in proximal tubule cells through a microtubule-dependent endocytic pathway. *Am J Physiol Renal Physiol,* 297, F1342-52.

LI, X. C., NAVAR, L. G., SHAO, Y. & ZHUO, J. L. (2007) Genetic deletion of AT1a receptors attenuates intracellular accumulation of ANG II in the kidney of AT1a receptor-deficient mice. *Am J Physiol Renal Physiol,* 293, F586-93.

LI, X. C. & ZHUO, J. L. (2007) Selective knockdown of AT1 receptors by RNA interference inhibits Val5-ANG II endocytosis and NHE-3 expression in immortalized rabbit proximal tubule cells. *Am J Physiol Cell Physiol,* 293, C367-78.

LI, X. C. & ZHUO, J. L. (2008a) In vivo regulation of AT1a receptor-mediated intracellular uptake of [125I]Val5-ANG II in the kidneys and adrenals of AT1a receptor-deficient mice. *Am J Physiol Renal Physiol,* 294, F293-302.

LI, X. C. & ZHUO, J. L. (2008b) Intracellular ANG II directly induces in vitro transcription of TGF-beta1, MCP-1, and NHE-3 mRNAs in isolated rat renal cortical nuclei via activation of nuclear AT1a receptors. *Am J Physiol Cell Physiol,* 294, C1034-45.

LICEA, H., WALTERS, M. R. & NAVAR, L. G. (2002) Renal nuclear angiotensin II receptors in normal and hypertensive rats. *Acta Physiol Hung,* 89, 427-38.

LILL, R., STUART, R. A., DRYGAS, M. E., NARGANG, F. E. & NEUPERT, W. (1992) Import of cytochrome c heme lyase into mitochondria: a novel pathway into the intermembrane space. *EMBO J,* 11, 449-56.

LIND, R. W., SWANSON, L. W. & GANTEN, D. (1985) Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. An immunohistochemical study. *Neuroendocrinology,* 40, 2-24.

LINDQUIST, S. (1986) The heat-shock response. *Annu Rev Biochem,* 55, 1151-91.

LIPPOLDT, A., BUNNEMANN, B., UEKI, A., ROSEN, L., CINTRA, A., HASSELROT, U., METZGER, R., HILGENFELDT, U., BROSNIHAN, B., GANTEN, D. & ET AL. (1994) On the plasticity of the cerebellar renin-angiotensin system: localization of components and effects of mechanical perturbation. *Brain Res,* 668, 144-59.

LOEW, L. M., TUFT, R. A., CARRINGTON, W. & FAY, F. S. (1993) Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria. *Biophys J,* 65, 2396-407.

MAILLOUX, R. J. & HARPER, M. E. (2011) Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radic Biol Med,* 51, 1106-15.

MARGOLIUS, H. S. (1996) Kallikreins and kinins. Molecular characteristics and cellular and tissue responses. *Diabetes,* 45 Suppl 1, S14-9.

MARIAPPAN, N., ELKS, C. M., HAQUE, M. & FRANCIS, J. (2012) Interaction of TNF with angiotensin II contributes to mitochondrial oxidative stress and cardiac damage in rats. *PLoS One,* 7, e46568.

MARKTANNER, R., NACKE, P., FEINDT, P., HOHLFELD, T., SCHIPKE, J. D. & GAMS, E. (2006) Delayed preconditioning via Angiotensin-converting enzyme inhibition: pros and cons from an experimental study. *Clin Exp Pharmacol Physiol,* 33, 787-92.

MATSUMURA, T., YOSHIHARA, H., JEFFS, R., TAKEI, Y., NUKINA, S., HIJIOKA, T., EVANS, R. K., KAUFFMAN, F. C. & THURMAN, R. G. (1992) Hormones increase oxygen uptake in periportal and pericentral regions of the liver lobule. *Am J Physiol,* 262, G645-50.

MATSUSAKA, T. & ICHIKAWA, I. (1997) Biological functions of angiotensin and its receptors. *Annu Rev Physiol,* 59, 395-412.

MEHTA, P. K. & GRIENDLING, K. K. (2007) Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol,* 292, C82-97.

MENARD, J. (1993) Anthology of the renin-angiotensin system: a one hundred reference approach to angiotensin II antagonists. *J Hypertens Suppl,* 11, S3-11.

MESSADI, E., VINCENT, M. P., GRIOL-CHARHBILI, V., MANDET, C., COLUCCI, J., KREGE, J. H., BRUNEVAL, P., BOUBY, N., SMITHIES, O., ALHENC-GELAS, F. & RICHER, C. (2010) Genetically determined angiotensin converting enzyme level and myocardial tolerance to ischemia. *FASEB J,* 24, 4691-700.

METZGER, R., BOHLE, R. M., PAULS, K., EICHNER, G., ALHENC-GELAS, F., DANILOV, S. M. & FRANKE, F. E. (1999) Angiotensin-converting enzyme in non-neoplastic kidney diseases. *Kidney Int,* 56, 1442-54.

MILLER, R. J. (1998) Mitochondria - the Kraken wakes! *Trends Neurosci,* 21, 95-7.

MITSUISHI, M., MIYASHITA, K., MURAKI, A. & ITOH, H. (2009) Angiotensin II reduces mitochondrial content in skeletal muscle and affects glycemic control. *Diabetes,* 58, 710-7.

MIYATA, T., TAGUCHI, T., UEHARA, M., ISAMI, S., KISHIKAWA, H., KANEKO, K., ARAKI, E. & SHICHIRI, M. (1998) Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B2 receptor in dog skeletal muscle and rat L6 myoblasts. *Eur J Endocrinol,* 138, 344-52.

MIZUNO, K., HOFFMAN, L. H., MCKENZIE, J. C. & INAGAMI, T. (1988) Presence of renin secretory granules in rat adrenal gland and stimulation of renin secretion by angiotensin II but not by adrenocorticotropin. *J Clin Invest,* 82, 1007-16.

MODRALL, J. G., SADJADI, J., BROSNIHAN, K. B., GALLAGHER, P. E., YU, C. H., KRAMER, G. L., BERNSTEIN, K. E. & CHAPPELL, M. C. (2004) Depletion of tissue angiotensin-converting enzyme differentially influences the intrarenal and urinary expression of angiotensin peptides. *Hypertension,* 43, 849-53.

MONTGOMERY, H. E., MARSHALL, R., HEMINGWAY, H., MYERSON, S., CLARKSON, P., DOLLERY, C., HAYWARD, M., HOLLIMAN, D. E., JUBB, M., WORLD, M., THOMAS, E. L., BRYNES, A. E., SAEED, N., BARNARD, M., BELL, J. D., PRASAD, K., RAYSON, M., TALMUD, P. J. & HUMPHRIES, S. E. (1998) Human gene for physical performance. *Nature,* 393, 221-2.

MORINELLI, T. A., RAYMOND, J. R., BALDYS, A., YANG, Q., LEE, M. H., LUTTRELL, L. & ULLIAN, M. E. (2007) Identification of a putative nuclear localization

sequence within ANG II AT(1A) receptor associated with nuclear activation. *Am J Physiol Cell Physiol,* 292, C1398-408.

MULLINS, J. J., BURT, D. W., WINDASS, J. D., MCTURK, P., GEORGE, H. & BRAMMAR, W. J. (1982) Molecular cloning of two distinct renin genes from the DBA/2 mouse. *EMBO J,* 1, 1461-6.

MULLINS, J. J., PETERS, J. & GANTEN, D. (1990) Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature,* 344, 541-4.

MURPHEY, L. J., GAINER, J. V., VAUGHAN, D. E. & BROWN, N. J. (2000) Angiotensin-converting enzyme insertion/deletion polymorphism modulates the human in vivo metabolism of bradykinin. *Circulation,* 102, 829-32.

MURPHY, J. E., PADILLA, B. E., HASDEMIR, B., COTTRELL, G. S. & BUNNETT, N. W. (2009) Endosomes: a legitimate platform for the signaling train. *Proc Natl Acad Sci U S A,* 106, 17615-22.

MYERSON, S., HEMINGWAY, H., BUDGET, R., MARTIN, J., HUMPHRIES, S. & MONTGOMERY, H. (1999) Human angiotensin I-converting enzyme gene and endurance performance. *J Appl Physiol,* 87, 1313-6.

MYHILL, N., LYNES, E. M., NANJI, J. A., BLAGOVESHCHENSKAYA, A. D., FEI, H., CARMINE SIMMEN, K., COOPER, T. J., THOMAS, G. & SIMMEN, T. (2008) The subcellular distribution of calnexin is mediated by PACS-2. *Mol Biol Cell,* 19, 2777-88.

NABI, A. H., KAGESHIMA, A., UDDIN, M. N., NAKAGAWA, T., PARK, E. Y. & SUZUKI, F. (2006) Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. *Int J Mol Med,* 18, 483-8.

NAVAR, L. G., MITCHELL, K. D., HARRISON-BERNARD, L. M., KOBORI, H. & NISHIYAMA, A. (2001) Intrarenal angiotensin II levels in normal and hypertensive states. *J Renin Angiotensin Aldosterone Syst,* 2, S176-S184.

NEGRE-SALVAYRE, A., HIRTZ, C., CARRERA, G., CAZENAVE, R., TROLY, M., SALVAYRE, R., PENICAUD, L. & CASTEILLA, L. (1997) A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J,* 11, 809-15.

NEUBAUER, B., MACHURA, K., CHEN, M., WEINSTEIN, L. S., OPPERMANN, M., SEQUEIRA-LOPEZ, M. L., GOMEZ, R. A., SCHNERMANN, J., CASTROP, H., KURTZ, A. & WAGNER, C. (2009) Development of vascular renin expression in the kidney critically depends on the cyclic AMP pathway. *Am J Physiol Renal Physiol,* 296, F1006-12.

NEUPERT, W. (1997) Protein import into mitochondria. *Annu Rev Biochem,* 66, 863-917.

NGUYEN, G. (2011) Renin, (pro)renin and receptor: an update. *Clin Sci (Lond),* 120, 169-78.

NGUYEN, G. (2010) The (pro)renin receptor in health and disease. *Ann Med,* 42, 13-8.

NGUYEN, G. & CONTREPAS, A. (2008) Physiology and pharmacology of the (pro)renin receptor. *Curr Opin Pharmacol,* 8, 127-32.

NGUYEN, G., DELARUE, F., BERROU, J., RONDEAU, E. & SRAER, J. D. (1996) Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int,* 50, 1897-903.

NGUYEN, G., DELARUE, F., BURCKLE, C., BOUZHIR, L., GILLER, T. & SRAER, J. D. (2002) Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest,* 109, 1417-27.

NICHOLSON, D. W., HERGERSBERG, C. & NEUPERT, W. (1988) Role of cytochrome c heme lyase in the import of cytochrome c into mitochondria. *J Biol Chem,* 263, 19034-42.

NITSCHKE, R., HENGER, A., RICKEN, S., GLOY, J., MULLER, V., GREGER, R. & PAVENSTADT, H. (2000) Angiotensin II increases the intracellular calcium activity in podocytes of the intact glomerulus. *Kidney Int,* 57, 41-9.

NOSTRAMO, R., TILLINGER, A., SAAVEDRA, J. M., KUMAR, A., PANDEY, V., SEROVA, L., KVETNANSKY, R. & SABBAN, E. L. (2012) Regulation of angiotensin II type 2 receptor gene expression in the adrenal medulla by acute and repeated immobilization stress. *J Endocrinol,* 215, 291-301.

PAIZIS, G., COOPER, M. E., SCHEMBRI, J. M., TIKELLIS, C., BURRELL, L. M. & ANGUS, P. W. (2002) Up-regulation of components of the renin-angiotensin system in the bile duct-ligated rat liver. *Gastroenterology,* 123, 1667-76.

PAIZIS, G., TIKELLIS, C., COOPER, M. E., SCHEMBRI, J. M., LEW, R. A., SMITH, A. I., SHAW, T., WARNER, F. J., ZUILLI, A., BURRELL, L. M. & ANGUS, P. W. (2005)

Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut,* 54, 1790-6.

PANDYA, J. D., NUKALA, V. N. & SULLIVAN, P. G. (2013) Concentration dependent effect of calcium on brain mitochondrial bioenergetics and oxidative stress parameters. *Front Neuroenergetics,* 5, 10.

PANG, S., CHUBB, A. J., SCHWAGER, S. L., EHLERS, M. R., STURROCK, E. D. & HOOPER, N. M. (2001) Roles of the juxtamembrane and extracellular domains of angiotensin-converting enzyme in ectodomain shedding. *Biochem J,* 358, 185-92.

PANTHIER, J. J., FOOTE, S., CHAMBRAUD, B., STROSBERG, A. D., CORVOL, P. & ROUGEON, F. (1982) Complete amino acid sequence and maturation of the mouse submaxillary gland renin precursor. *Nature,* 298, 90-2.

PAUL, M., POYAN MEHR, A. & KREUTZ, R. (2006) Physiology of local renin-angiotensin systems. *Physiol Rev,* 86, 747-803.

PEACH, M. J. (1977) Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol Rev,* 57, 313-70.

PETERS, J., FARRENKOPF, R., CLAUSMEYER, S., ZIMMER, J., KANTACHUVESIRI, S., SHARP, M. G. & MULLINS, J. J. (2002) Functional significance of prorenin internalization in the rat heart. *Circ Res,* 90, 1135-41.

PETERS, J. & GANTEN, D. (1998) Adrenal renin expression and its role in ren-2 transgenic rats TGR(mREN2)27. *Horm Metab Res,* 30, 350-4.

PETERS, J., KRANZLIN, B., SCHAEFFER, S., ZIMMER, J., RESCH, S., BACHMANN, S., GRETZ, N. & HACKENTHAL, E. (1996) Presence of renin within intramitochondrial dense bodies of the rat adrenal cortex. *Am J Physiol,* 271, E439-50.

PETERS, J., MUNTER, K., BADER, M., HACKENTHAL, E., MULLINS, J. J. & GANTEN, D. (1993) Increased adrenal renin in transgenic hypertensive rats, TGR(mREN2)27, and its regulation by cAMP, angiotensin II, and calcium. *J Clin Invest,* 91, 742-7.

PETERS, J., OBERMULLER, N., WOYTH, A., PETERS, B., MASER-GLUTH, C., KRANZLIN, B. & GRETZ, N. (1999) Losartan and angiotensin II inhibit aldosterone

production in anephric rats via different actions on the intraadrenal renin-angiotensin system. *Endocrinology,* 140, 675-82.

PFANNER, N., HOEBEN, P., TROPSCHUG, M. & NEUPERT, W. (1987) The carboxyl-terminal two-thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria. *J Biol Chem,* 262, 14851-4.

PIERUZZI, F., ABASSI, Z. A. & KEISER, H. R. (1995) Expression of renin-angiotensin system components in the heart, kidneys, and lungs of rats with experimental heart failure. *Circulation,* 92, 3105-12.

PIOTRKOWSKI, B., FRAGA, C. G. & DE CAVANAGH, E. M. (2007) Mitochondrial function and nitric oxide metabolism are modified by enalapril treatment in rat kidney. *Am J Physiol Regul Integr Comp Physiol,* 292, R1494-501.

PIOTRKOWSKI, B., KOCH, O. R., DE CAVANAGH, E. M. & FRAGA, C. G. (2009) Cardiac mitochondrial function and tissue remodelling are improved by a non-antihypertensive dose of enalapril in spontaneously hypertensive rats. *Free Radic Res,* 43, 390-9.

POMPELLA, A., VISVIKIS, A., PAOLICCHI, A., DE TATA, V. & CASINI, A. F. (2003) The changing faces of glutathione, a cellular protagonist. *Biochem Pharmacol,* 66, 1499-503.

PORTUGAL, J. & WARING, M. J. (1988) Assignment of DNA binding sites for 4',6-diamidine-2-phenylindole and bisbenzimide (Hoechst 33258). A comparative footprinting study. *Biochim Biophys Acta,* 949, 158-68.

PRATT, R. E., OUELLETTE, A. J. & DZAU, V. J. (1983) Biosynthesis of renin: multiplicity of active and intermediate forms. *Proc Natl Acad Sci U S A,* 80, 6809-13.

PRESCOTT, G., SILVERSIDES, D. W. & REUDELHUBER, T. L. (2002) Tissue activity of circulating prorenin. *Am J Hypertens,* 15, 280-5.

PURI, P. L., AVANTAGGIATI, M. L., BURGIO, V. L., CHIRILLO, P., COLLEPARDO, D., NATOLI, G., BALSANO, C. & LEVRERO, M. (1995) Reactive oxygen intermediates mediate angiotensin II-induced c-Jun.c-Fos heterodimer DNA binding activity and proliferative hypertrophic responses in myogenic cells. *J Biol Chem,* 270, 22129-34.

RABILLOUD, T., VUILLARD, L., GILLY, C. & LAWRENCE, J. J. (1994) Silver-staining of proteins in polyacrylamide gels: a general overview. *Cell Mol Biol (Noisy-le-grand),* 40, 57-75.

RACZ, K., PINET, F., GASC, J. M., GUYENE, T. T. & CORVOL, P. (1992) Coexpression of renin, angiotensinogen, and their messenger ribonucleic acids in adrenal tissues. *J Clin Endocrinol Metab,* 75, 730-7.

RAJAGOPAL, K., WHALEN, E. J., VIOLIN, J. D., STIBER, J. A., ROSENBERG, P. B., PREMONT, R. T., COFFMAN, T. M., ROCKMAN, H. A. & LEFKOWITZ, R. J. (2006) Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci U S A,* 103, 16284-9.

RAMMOS, G., TSEKE, P. & ZIAKKA, S. (2008) Vitamin D, the renin-angiotensin system, and insulin resistance. *Int Urol Nephrol,* 40, 419-26.

RE, R. N. (1984) Cellular biology of the renin-angiotensin systems. *Arch Intern Med,* 144, 2037-41.

RE, R. N. (2003) Cardiac angiotensin II: an intracrine hormone? *Am J Hypertens,* 16, 426-7.

RE, R. N. & COOK, J. L. (2010) The mitochondrial component of intracrine action. *Am J Physiol Heart Circ Physiol,* 299, H577-83.

RE, R. N., MACPHEE, A. A. & FALLON, J. T. (1981) Specific nuclear binding of angiotensin II by rat liver and spleen nuclei. *Clin Sci (Lond),* 61 Suppl 7, 245s-247s.

REAMS, G. P. (1992) Angiotensin-converting enzyme in renal and cerebral tissue and implications for successful blood pressure management. *Am J Cardiol,* 69, 59C-64C.

REDDING, K. M., CHEN, B. L., SINGH, A., RE, R. N., NAVAR, L. G., SETH, D. M., SIGMUND, C. D., TANG, W. W. & COOK, J. L. (2010) Transgenic mice expressing an intracellular fluorescent fusion of angiotensin II demonstrate renal thrombotic microangiopathy and elevated blood pressure. *Am J Physiol Heart Circ Physiol,* 298, H1807-18.

REGULSKA, K., STANISZ, B. & REGULSKI, M. (2013) The Renin-Angiotensin System As A Target of Novel Anticancer Therapy. *Curr Pharm Des*, 19, 7103-25.

REHLING, P., WIEDEMANN, N., PFANNER, N. & TRUSCOTT, K. N. (2001) The mitochondrial import machinery for preproteins. *Crit Rev Biochem Mol Biol,* 36, 291-336.

REID, I. A., MORRIS, B. J. & GANONG, W. F. (1978) The renin-angiotensin system. *Annu Rev Physiol,* 40, 377-410.

RIGAT, B., HUBERT, C., ALHENC-GELAS, F., CAMBIEN, F., CORVOL, P. & SOUBRIER, F. (1990) An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest,* 86, 1343-6.

RIZZUTO, R., MARCHI, S., BONORA, M., AGUIARI, P., BONONI, A., DE STEFANI, D., GIORGI, C., LEO, S., RIMESSI, A., SIVIERO, R., ZECCHINI, E. & PINTON, P. (2009) Ca(2+) transfer from the ER to mitochondria: when, how and why. *Biochim Biophys Acta,* 1787, 1342-51.

RIZZUTO, R., PINTON, P., CARRINGTON, W., FAY, F. S., FOGARTY, K. E., LIFSHITZ, L. M., TUFT, R. A. & POZZAN, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science,* 280, 1763-6.

ROBERTSON, A. L., JR. & KHAIRALLAH, P. A. (1971a) Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle. *Science,* 172, 1138-9.

ROBERTSON, A. L., JR. & KHAIRALLAH, P. A. (1971b) Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle. *Science,* 172, 1138-9.

ROCKEY, D. (1997) The cellular pathogenesis of portal hypertension: stellate cell contractility, endothelin, and nitric oxide. *Hepatology,* 25, 2-5.

ROHRWASSER, A., MORGAN, T., DILLON, H. F., ZHAO, L., CALLAWAY, C. W., HILLAS, E., ZHANG, S., CHENG, T., INAGAMI, T., WARD, K., TERREROS, D. A. & LALOUEL, J. M. (1999) Elements of a paracrine tubular renin-angiotensin system along the entire nephron. *Hypertension,* 34, 1265-74.

ROISE, D. & SCHATZ, G. (1988) Mitochondrial presequences. *J Biol Chem,* 263, 4509-11.

RONCHI, F. A., ANDRADE, M. C., CARMONA, A. K., KRIEGER, J. E. & CASARINI, D. E. (2005) N-domain angiotensin-converting enzyme isoform expression in tissues of Wistar and spontaneously hypertensive rats. *J Hypertens,* 23, 1869-78.

RONCHI, F. A., IRIGOYEN, M. C. & CASARINI, D. E. (2007) Association of somatic and N-domain angiotensin-converting enzymes from Wistar rat tissue with renal dysfunction in diabetes mellitus. *J Renin Angiotensin Aldosterone Syst,* 8, 34-41.

RONG, P., BERKA, J. L., KELLY, D. J., ALCORN, D. & SKINNER, S. L. (1994) Renin processing and secretion in adrenal and retina of transgenic (mREN-2)27 rats. *Kidney Int,* 46, 1583-7.

ROSIVALL, L. & NAVAR, L. G. (1983) Effects on renal hemodynamics of intra-arterial infusions of angiotensins I and II. *Am J Physiol,* 245, F181-7.

ROUSSET, S., ALVES-GUERRA, M. C., MOZO, J., MIROUX, B., CASSARD-DOULCIER, A. M., BOUILLAUD, F. & RICQUIER, D. (2004) The biology of mitochondrial uncoupling proteins. *Diabetes,* 53 Suppl 1, S130-5.

ROWLAND, A. A. & VOELTZ, G. K. (2012) Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat Rev Mol Cell Biol,* 13, 607-25.

RUBIO, M. A., RINEHART, J. J., KRETT, B., DUVEZIN-CAUBET, S., REICHERT, A. S., SOLL, D. & ALFONZO, J. D. (2008) Mammalian mitochondria have the innate ability to import tRNAs by a mechanism distinct from protein import. *Proc Natl Acad Sci U S A,* 105, 9186-91.

RUSINOL, A. E., CUI, Z., CHEN, M. H. & VANCE, J. E. (1994) A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins. *J Biol Chem,* 269, 27494-502.

SADJADI, J., KRAMER, G. L., YU, C. H., WELBORN, M. B., 3RD & MODRALL, J. G. (2005) Angiotensin II exerts positive feedback on the intrarenal renin-angiotensin system by an angiotensin converting enzyme-dependent mechanism. *J Surg Res,* 129, 272-7.

SAMPAIO, W. O., SOUZA DOS SANTOS, R. A., FARIA-SILVA, R., DA MATA MACHADO, L. T., SCHIFFRIN, E. L. & TOUYZ, R. M. (2007) Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension,* 49, 185-92.

SANDERS, P. M., RUSSELL, S. T. & TISDALE, M. J. (2005) Angiotensin II directly induces muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway and may play a role in cancer cachexia. *Br J Cancer,* 93, 425-34.

SANTOS, R. A., SIMOES E SILVA, A. C., MARIC, C., SILVA, D. M., MACHADO, R. P., DE BUHR, I., HERINGER-WALTHER, S., PINHEIRO, S. V., LOPES, M. T., BADER, M., MENDES, E. P., LEMOS, V. S., CAMPAGNOLE-SANTOS, M. J., SCHULTHEISS,

H. P., SPETH, R. & WALTHER, T. (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A,* 100, 8258-63.

SAOTOME, M., SAFIULINA, D., SZABADKAI, G., DAS, S., FRANSSON, A., ASPENSTROM, P., RIZZUTO, R. & HAJNOCZKY, G. (2008) Bidirectional Ca2+-dependent control of mitochondrial dynamics by the Miro GTPase. *Proc Natl Acad Sci U S A,* 105, 20728-33.

SARIS, J. J., DERKX, F. H., LAMERS, J. M., SAXENA, P. R., SCHALEKAMP, M. A. & DANSER, A. H. (2001) Cardiomyocytes bind and activate native human prorenin : role of soluble mannose 6-phosphate receptors. *Hypertension,* 37, 710-5.

SARIS, J. J., T HOEN, P. A., GARRELDS, I. M., DEKKERS, D. H., DEN DUNNEN, J. T., LAMERS, J. M. & JAN DANSER, A. H. (2006) Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension,* 48, 564-71.

SCHMIDT, O., PFANNER, N. & MEISINGER, C. (2010) Mitochondrial protein import: from proteomics to functional mechanisms. *Nat Rev Mol Cell Biol,* 11, 655-67.

SEALEY, J. E., GLORIOSO, N., ITSKOVITZ, J. & LARAGH, J. H. (1986) Prorenin as a reproductive hormone. New form of the renin system. *Am J Med,* 81, 1041-6.

SECHI, S. & CHAIT, B. T. (1998) Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. *Anal Chem,* 70, 5150-8.

SHAO, W., SETH, D. M. & NAVAR, L. G. (2009) Augmentation of endogenous intrarenal angiotensin II levels in Val5-ANG II-infused rats. *Am J Physiol Renal Physiol,* 296, F1067-71.

SHARP, M. G., FETTES, D., BROOKER, G., CLARK, A. F., PETERS, J., FLEMING, S. & MULLINS, J. J. (1996) Targeted inactivation of the Ren-2 gene in mice. *Hypertension,* 28, 1126-31.

SHERROD, M., LIU, X., ZHANG, X. & SIGMUND, C. D. (2005) Nuclear localization of angiotensinogen in astrocytes. *Am J Physiol Regul Integr Comp Physiol,* 288, R539-46.

SHOSHAN-BARMATZ, V., DE PINTO, V., ZWECKSTETTER, M., RAVIV, Z., KEINAN, N. & ARBEL, N. (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol Aspects Med,* 31, 227-85.

SHUM, M., PINARD, S., GUIMOND, M. O., LABBE, S. M., ROBERGE, C., BAILLARGEON, J. P., LANGLOIS, M. F., ALTERMAN, M., WALLINDER, C., HALLBERG, A., CARPENTIER, A. C. & GALLO-PAYET, N. (2013) Angiotensin II type 2 receptor promotes adipocyte differentiation and restores adipocyte size in high-fat/high-fructose diet-induced insulin resistance in rats. *Am J Physiol Endocrinol Metab,* 304, E197-210.

SIHN, G., ROUSSELLE, A., VILIANOVITCH, L., BURCKLE, C. & BADER, M. (2010) Physiology of the (pro)renin receptor: Wnt of change? *Kidney Int,* 78, 246-56.

SIMS, N. R. & ANDERSON, M. F. (2008) Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. *Nat Protoc,* 3, 1228-39.

SINGH, V. P., LE, B., BHAT, V. B., BAKER, K. M. & KUMAR, R. (2007) High-glucose-induced regulation of intracellular ANG II synthesis and nuclear redistribution in cardiac myocytes. *Am J Physiol Heart Circ Physiol,* 293, H939-48.

SINGH, V. P., LE, B., KHODE, R., BAKER, K. M. & KUMAR, R. (2008) Intracellular angiotensin II production in diabetic rats is correlated with cardiomyocyte apoptosis, oxidative stress, and cardiac fibrosis. *Diabetes,* 57, 3297-306.

SIRETT, N. E., MCLEAN, A. S., BRAY, J. J. & HUBBARD, J. I. (1977) Distribution of angiotensin II receptors in rat brain. *Brain Res,* 122, 299-312.

SLOT, J. W., POSTHUMA, G., CHANG, L. Y., CRAPO, J. D. & GEUZE, H. J. (1989) Quantitative assessment of immuno-gold labeling in cryosections. *Am J Anat,* 185, 271-81.

SOINILA, S., MPITSOS, G. J. & SOINILA, J. (1992) Immunohistochemistry of enkephalins: model studies on hapten-carrier conjugates and fixation methods. *J Histochem Cytochem,* 40, 231-9.

SONG, K., ZHUO, J., CHAI, S. Y. & MENDELSOHN, F. A. (1992) A new method to localize active renin in tissues by autoradiography: application to dog kidney. *Kidney Int,* 42, 639-46.

SOOKOIAN, S., FERNANDEZ, M. A. & CASTANO, G. (2005) Effects of six months losartan administration on liver fibrosis in chronic hepatitis C patients: a pilot study. *World J Gastroenterol,* 11, 7560-3.

SPAT, A. & HUNYADY, L. (2004) Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol Rev,* 84, 489-539.

SPEICHER, K. D., KOLBAS, O., HARPER, S. & SPEICHER, D. W. (2000) Systematic analysis of peptide recoveries from in-gel digestions for protein identifications in proteome studies. *J Biomol Tech,* 11, 74-86.

SPETH, R. C., DAUBERT, D. L. & GROVE, K. L. (1999) Angiotensin II: a reproductive hormone too? *Regul Pept,* 79, 25-40.

STURROCK, E. D., NATESH, R., VAN ROOYEN, J. M. & ACHARYA, K. R. (2004) Structure of angiotensin I-converting enzyme. *Cell Mol Life Sci,* 61, 2677-86.

SUMBALOVA, Z., KUCHARSKA, J. & KRISTEK, F. (2010) Losartan improved respiratory function and coenzyme Q content in brain mitochondria of young spontaneously hypertensive rats. *Cell Mol Neurobiol,* 30, 751-8.

SUZUKI, Y., RUIZ-ORTEGA, M., GOMEZ-GUERRERO, C., TOMINO, Y. & EGIDO, J. (2003) Angiotensin II, the immune system and renal diseases: another road for RAS? *Nephrol Dial Transplant,* 18, 1423-6.

SZABADKAI, G., BIANCHI, K., VARNAI, P., DE STEFANI, D., WIECKOWSKI, M. R., CAVAGNA, D., NAGY, A. I., BALLA, T. & RIZZUTO, R. (2006) Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. *J Cell Biol,* 175, 901-11.

SZABADKAI, G. & RIZZUTO, R. (2004) Participation of endoplasmic reticulum and mitochondrial calcium handling in apoptosis: more than just neighborhood? *FEBS Lett,* 567, 111-5.

TADEVOSYAN, A., MAGUY, A., VILLENEUVE, L. R., BABIN, J., BONNEFOY, A., ALLEN, B. G. & NATTEL, S. (2010) Nuclear-delimited angiotensin receptor-mediated signaling regulates cardiomyocyte gene expression. *J Biol Chem,* 285, 22338-49.

TASKIN, E., OZDOGAN, K., KUNDUZ KINDAP, E. & DURSUN, N. (2014) The restoration of kidney mitochondria function by inhibition of angiotensin-II production in rats with acute adriamycin-induced nephrotoxicity. *Ren Fail*.

TAUGNER, R., HACKENTHAL, E., NOBILING, R., HARLACHER, M. & REB, G. (1981) The distribution of renin in the different segments of the renal arterial tree: immunocytochemical investigation in the mouse kidney. *Histochemistry,* 73, 75-88.

TERUI, Y., SAITO, T., WATANABE, H., TOGASHI, H., KAWATA, S., KAMADA, Y. & SAKUTA, S. (2002) Effect of angiotensin receptor antagonist on liver fibrosis in early stages of chronic hepatitis C. *Hepatology,* 36, 1022.

THEKKUMKARA, T. & LINAS, S. L. (2002) Role of internalization in AT(1A) receptor function in proximal tubule epithelium. *Am J Physiol Renal Physiol,* 282, F623-9.

THOMAS, W. G., THEKKUMKARA, T. J. & BAKER, K. M. (1996) Molecular mechanisms of angiotensin II (AT1a) receptor endocytosis. *Clin Exp Pharmacol Physiol,* 23 Suppl 3, S74-80.

THOMPSON, J., RAITT, J., HUTCHINGS, L., DRENOS, F., BJARGO, E., LOSET, A., GROCOTT, M. & MONTGOMERY, H. (2007) Angiotensin-converting enzyme genotype and successful ascent to extreme high altitude. *High Alt Med Biol,* 8, 278-85.

TIKELLIS, C., BIALKOWSKI, K., PETE, J., SHEEHY, K., SU, Q., JOHNSTON, C., COOPER, M. E. & THOMAS, M. C. (2008) ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. *Diabetes,* 57, 1018-25.

TIMMERMANS, P. B. & SMITH, R. D. (1994) Angiotensin II receptor subtypes: selective antagonists and functional correlates. *Eur Heart J,* 15 Suppl D, 79-87.

TIMMERMANS, P. B., WONG, P. C., CHIU, A. T., HERBLIN, W. F., BENFIELD, P., CARINI, D. J., LEE, R. J., WEXLER, R. R., SAYE, J. A. & SMITH, R. D. (1993) Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev,* 45, 205-51.

TIPNIS, S. R., HOOPER, N. M., HYDE, R., KARRAN, E., CHRISTIE, G. & TURNER, A. J. (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem,* 275, 33238-43.

TO, M. S., AROMATARIS, E. C., CASTRO, J., ROBERTS, M. L., BARRITT, G. J. & RYCHKOV, G. Y. (2010) Mitochondrial uncoupler FCCP activates proton conductance but does not block store-operated Ca(2+) current in liver cells. *Arch Biochem Biophys,* 495, 152-8.

TOBLLI, J. E., MUNOZ, M. C., CAO, G., MELLA, J., PEREYRA, L. & MASTAI, R. (2008) ACE inhibition and AT1 receptor blockade prevent fatty liver and fibrosis in obese Zucker rats. *Obesity (Silver Spring),* 16, 770-6.

TOUYZ, R. M. (2005) Reactive oxygen species as mediators of calcium signaling by angiotensin II: implications in vascular physiology and pathophysiology. *Antioxid Redox Signal,* 7, 1302-14.

TOUYZ, R. M. & SCHIFFRIN, E. L. (2000) Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev,* 52, 639-72.

TSIANOS, G., ELEFTHERIOU, K. I., HAWE, E., WOOLRICH, L., WATT, M., WATT, I., PEACOCK, A., MONTGOMERY, H. & GRANT, S. (2005) Performance at altitude and angiotensin I-converting enzyme genotype. *Eur J Appl Physiol,* 93, 630-3.

TSIANOS, G., WOOLRICH-BURT, L., AITCHISON, T., PEACOCK, A., WATT, M., MONTGOMERY, H., WATT, I. & GRANT, S. (2006) Factors affecting a climber's ability to ascend Mont Blanc. *Eur J Appl Physiol,* 96, 32-6.

TUNCER, I., OZBEK, H., UGRAS, S. & BAYRAM, I. (2003) Anti-fibrogenic effects of captopril and candesartan cilexetil on the hepatic fibrosis development in rat. The effect of AT1-R blocker on the hepatic fibrosis. *Exp Toxicol Pathol,* 55, 159-66.

TURNER, A. J., TIPNIS, S. R., GUY, J. L., RICE, G. & HOOPER, N. M. (2002) ACEH/ACE2 is a novel mammalian metallocarboxypeptidase and a homologue of angiotensin-converting enzyme insensitive to ACE inhibitors. *Can J Physiol Pharmacol,* 80, 346-53.

UEKI, M., KODA, M., YAMAMOTO, S., MATSUNAGA, Y. & MURAWAKI, Y. (2006) Preventive and therapeutic effects of angiotensin II type 1 receptor blocker on hepatic fibrosis induced by bile duct ligation in rats. *J Gastroenterol,* 41, 996-1004.

VAN KATS, J. P., CHAI, W., DUNCKER, D. J., SCHALEKAMP, M. A. & DANSER, A. H. (2005) Adrenal angiotensin: origin and site of generation. *Am J Hypertens,* 18, 1104-10.

VAN KATS, J. P., SCHALEKAMP, M. A., VERDOUW, P. D., DUNCKER, D. J. & DANSER, A. H. (2001) Intrarenal angiotensin II: interstitial and cellular levels and site of production. *Kidney Int,* 60, 2311-7.

VAN KESTEREN, C. A., DANSER, A. H., DERKX, F. H., DEKKERS, D. H., LAMERS, J. M., SAXENA, P. R. & SCHALEKAMP, M. A. (1997) Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cardiac cells. *Hypertension,* 30, 1389-96.

VANCE, J. E. (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J Biol Chem,* 265, 7248-56.

VATASSERY, G. T., SANTACRUZ, K. S., DEMASTER, E. G., QUACH, H. T. & SMITH, W. E. (2004) Oxidative stress and inhibition of oxidative phosphorylation induced by peroxynitrite and nitrite in rat brain subcellular fractions. *Neurochem Int,* 45, 963-70.

VICKERS, C., HALES, P., KAUSHIK, V., DICK, L., GAVIN, J., TANG, J., GODBOUT, K., PARSONS, T., BARONAS, E., HSIEH, F., ACTON, S., PATANE, M., NICHOLS, A. & TUMMINO, P. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem,* 277, 14838-43.

VIGANO, A., TRUTSCHNIGG, B., KILGOUR, R. D., HAMEL, N., HORNBY, L., LUCAR, E., FOULKES, W., TREMBLAY, M. L. & MORAIS, J. A. (2009) Relationship between angiotensin-converting enzyme gene polymorphism and body composition, functional performance, and blood biomarkers in advanced cancer patients. *Clin Cancer Res,* 15, 2442-7.

VON HEIJNE, G., STEPPUHN, J. & HERRMANN, R. G. (1989) Domain structure of mitochondrial and chloroplast targeting peptides. *Eur J Biochem,* 180, 535-45.

VON THUN, A. M., VARI, R. C., EL-DAHR, S. S. & NAVAR, L. G. (1994) Augmentation of intrarenal angiotensin II levels by chronic angiotensin II infusion. *Am J Physiol,* 266, F120-8.

WALTERS, P. E., GASPARI, T. A. & WIDDOP, R. E. (2005) Angiotensin-(1-7) acts as a vasodepressor agent via angiotensin II type 2 receptors in conscious rats. *Hypertension,* 45, 960-6.

WANG, T. & CHAN, Y. L. (1991) The role of phosphoinositide turnover in mediating the biphasic effect of angiotensin II on renal tubular transport. *J Pharmacol Exp Ther,* 256, 309-17.

WANKA, H., KESSLER, N., ELLMER, J., ENDLICH, N., PETERS, B. S., CLAUSMEYER, S. & PETERS, J. (2009) Cytosolic renin is targeted to mitochondria and induces apoptosis in H9c2 rat cardiomyoblasts. *J Cell Mol Med,* 13, 2926-37.

WEI, Y., CLARK, S. E., THYFAULT, J. P., UPTERGROVE, G. M., LI, W., WHALEY-CONNELL, A. T., FERRARIO, C. M., SOWERS, J. R. & IBDAH, J. A. (2009) Oxidative stress-mediated mitochondrial dysfunction contributes to angiotensin II-induced nonalcoholic fatty liver disease in transgenic Ren2 rats. *Am J Pathol,* 174, 1329-37.

WILLIAMS, A. G., DHAMRAIT, S. S., WOOTTON, P. T., DAY, S. H., HAWE, E., PAYNE, J. R., MYERSON, S. G., WORLD, M., BUDGETT, R., HUMPHRIES, S. E. & MONTGOMERY, H. E. (2004) Bradykinin receptor gene variant and human physical performance. *J Appl Physiol,* 96, 938-42.

WILLIAMS, G. H. & HOLLENBERG, N. K. (1991) Functional derangements in the regulation of aldosterone secretion in hypertension. *Hypertension,* 18, III143-9.

WONG, T. P., DEBNAM, E. S. & LEUNG, P. S. (2007) Involvement of an enterocyte renin-angiotensin system in the local control of SGLT1-dependent glucose uptake across the rat small intestinal brush border membrane. *J Physiol,* 584, 613-23.

WOODS, D. R., WORLD, M., RAYSON, M. P., WILLIAMS, A. G., JUBB, M., JAMSHIDI, Y., HAYWARD, M., MARY, D. A., HUMPHRIES, S. E. & MONTGOMERY, H. E. (2002) Endurance enhancement related to the human angiotensin I-converting enzyme I-D polymorphism is not due to differences in the cardiorespiratory response to training. *Eur J Appl Physiol,* 86, 240-4.

XIA, H. & LAZARTIGUES, E. (2008) Angiotensin-converting enzyme 2 in the brain: properties and future directions. *J Neurochem,* 107, 1482-94.

XIA, Y., BUJA, L. M. & MCMILLIN, J. B. (1998) Activation of the cytochrome c gene by electrical stimulation in neonatal rat cardiac myocytes. Role of NRF-1 and c-Jun. *J Biol Chem,* 273, 12593-8.

YAMAGUCHI, F., KAWANA, K., TANONAKA, K., KAMANO, I., IGARASHI, T., GEN, E., FUJIMOTO, Y., MAKI, T., SANBE, A., NASA, Y. & TAKEO, S. (1999) Improvement of exercise capacity of rats with chronic heart failure by long-term treatment with trandolapril. *Br J Pharmacol,* 126, 1585-92.

YANG, H., ZENG, X. J., WANG, H. X., ZHANG, L. K., DONG, X. L., GUO, S., DU, J., LI, H. H. & TANG, C. S. (2011) Angiotensin IV protects against angiotensin II-induced cardiac injury via AT4 receptor. *Peptides,* 32, 2108-15.

YANG, L., BATALLER, R., DULYX, J., COFFMAN, T. M., GINES, P., RIPPE, R. A. & BRENNER, D. A. (2005) Attenuated hepatic inflammation and fibrosis in angiotensin type 1a receptor deficient mice. *J Hepatol,* 43, 317-23.

YIN, J. X., YANG, R. F., LI, S., RENSHAW, A. O., LI, Y. L., SCHULTZ, H. D. & ZIMMERMAN, M. C. (2010) Mitochondria-produced superoxide mediates angiotensin II-induced inhibition of neuronal potassium current. *Am J Physiol Cell Physiol,* 298, C857-65.

YOGEV, O. & PINES, O. (2011) Dual targeting of mitochondrial proteins: mechanism, regulation and function. *Biochim Biophys Acta,* 1808, 1012-20.

YOKOHAMA, S., TOKUSASHI, Y., NAKAMURA, K., TAMAKI, Y., OKAMOTO, S., OKADA, M., ASO, K., HASEGAWA, T., AOSHIMA, M., MIYOKAWA, N., HANEDA, M. & YONEDA, M. (2006) Inhibitory effect of angiotensin II receptor antagonist on hepatic stellate cell activation in non-alcoholic steatohepatitis. *World J Gastroenterol,* 12, 322-6.

YOSHIJI, H., NOGUCHI, R. & FUKUI, H. (2005) Combined effect of an ACE inhibitor, perindopril, and interferon on liver fibrosis markers in patients with chronic hepatitis C. *J Gastroenterol,* 40, 215-6.

YOSHIJI, H., NOGUCHI, R., KOJIMA, H., IKENAKA, Y., KITADE, M., KAJI, K., UEMURA, M., YAMAO, J., FUJIMOTO, M., YAMAZAKI, M., TOYOHARA, M., MITORO, A. & FUKUI, H. (2006) Interferon augments the anti-fibrotic activity of an angiotensin-converting enzyme inhibitor in patients with refractory chronic hepatitis C. *World J Gastroenterol,* 12, 6786-91.

ZHANG, G. X., LU, X. M., KIMURA, S. & NISHIYAMA, A. (2007) Role of mitochondria in angiotensin II-induced reactive oxygen species and mitogen-activated protein kinase activation. *Cardiovasc Res,* 76, 204-12.

ZHANG, H., SCHMEISSER, A., GARLICHS, C. D., PLOTZE, K., DAMME, U., MUGGE, A. & DANIEL, W. G. (1999a) Angiotensin II-induced superoxide anion generation in human vascular endothelial cells: role of membrane-bound NADH-/NADPH-oxidases. *Cardiovasc Res,* 44, 215-22.

ZHANG, J., BARAK, L. S., ANBORGH, P. H., LAPORTE, S. A., CARON, M. G. & FERGUSON, S. S. (1999b) Cellular trafficking of G protein-coupled receptor/beta-arrestin endocytic complexes. *J Biol Chem,* 274, 10999-1006.

ZHANG, J., FERGUSON, S. S., BARAK, L. S., ABER, M. J., GIROS, B., LEFKOWITZ, R. J. & CARON, M. G. (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptors Channels,* 5, 193-9.

ZHAO, J., LIU, J., PANG, X., WANG, S., WU, D., ZHANG, X. & FENG, L. (2013) Angiotensin II induces C-reactive protein expression via AT1-ROS-MAPK-NF-kappaB signal pathway in hepatocytes. *Cell Physiol Biochem,* 32, 569-80.

ZHONG, J. C., YU, X. Y., LIN, Q. X., LI, X. H., HUANG, X. Z., XIAO, D. Z. & LIN, S. G. (2008) Enhanced angiotensin converting enzyme 2 regulates the insulin/Akt signalling pathway by blockade of macrophage migration inhibitory factor expression. *Br J Pharmacol,* 153, 66-74.

ZHOU, A., CARRELL, R. W., MURPHY, M. P., WEI, Z., YAN, Y., STANLEY, P. L., STEIN, P. E., BROUGHTON PIPKIN, F. & READ, R. J. (2010) A redox switch in angiotensinogen modulates angiotensin release. *Nature,* 468, 108-11.

ZHUO, J., ANDERSON, W. P., SONG, K. & MENDELSOHN, F. A. (1996) Autoradiographic localization of active renin in the juxtaglomerular apparatus of the dog kidney: effects of sodium intake. *Clin Exp Pharmacol Physiol,* 23, 291-8.

ZHUO, J., MOELLER, I., JENKINS, T., CHAI, S. Y., ALLEN, A. M., OHISHI, M. & MENDELSOHN, F. A. (1998) Mapping tissue angiotensin-converting enzyme and angiotensin AT1, AT2 and AT4 receptors. *J Hypertens,* 16, 2027-37.

ZHUO, J., THOMAS, D., HARRIS, P. J. & SKINNER, S. L. (1992) The role of endogenous angiotensin II in the regulation of renal haemodynamics and proximal fluid reabsorption in the rat. *J Physiol,* 453, 1-13.

ZHUO, J. L. (2006) Intracrine renin and angiotensin II: a novel role in cardiovascular and renal cellular regulation. *J Hypertens,* 24, 1017-20.

ZHUO, J. L., CARRETERO, O. A. & LI, X. C. (2006a) Effects of AT1 receptor-mediated endocytosis of extracellular Ang II on activation of nuclear factor-kappa B in proximal tubule cells. *Ann N Y Acad Sci,* 1091, 336-45.

ZHUO, J. L., IMIG, J. D., HAMMOND, T. G., ORENGO, S., BENES, E. & NAVAR, L. G. (2002) Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT(1) receptor. *Hypertension,* 39, 116-21.

ZHUO, J. L. & LI, X. C. (2007) Novel roles of intracrine angiotensin II and signalling mechanisms in kidney cells. *J Renin Angiotensin Aldosterone Syst,* 8, 23-33.

ZHUO, J. L., LI, X. C., GARVIN, J. L., NAVAR, L. G. & CARRETERO, O. A. (2006b) Intracellular ANG II induces cytosolic Ca2+ mobilization by stimulating intracellular AT1 receptors in proximal tubule cells. *Am J Physiol Renal Physiol,* 290, F1382-90.

ZOU, L. X., IMIG, J. D., HYMEL, A. & NAVAR, L. G. (1998) Renal uptake of circulating angiotensin II in Val5-angiotensin II infused rats is mediated by AT1 receptor. *Am J Hypertens,* 11, 570-8.

APPENDICES

APP1. Experimental Working Solutions

APP1. a. Krebs-Ringer Buffer (KRB)

KRB is a buffer solution utilised for maintenance of osmotic balance and pH within cells, as well as providing access to water and inorganic ions. 1 L of KRB is made up by dissolving the following in 1 L of distilled water at pH 7.4 and storing at 4 ˚C (see Table APP1. a.).

Ion/Reagent	Molecular Weight	Final Concentration
NaCl	58.44	125 mM
KCl	74.55	5 mM
$Na_3PO_4.12H2O$	380.12	1 mM
$MgSO_4.7H2O$	246.48	1 mM
Glucose	180.16	5 mM
HEPES (Organic chemical buffer)	238.3	20 mM

Table APP1. a. The ingredients for KRB Solution

APP1. b. Phosphate-Buffered Saline (PBS) (Invitrogen (Cat. No. 20012-068)); Carlsbad, CA, USA)

PBS is an isotonic water-based salt solution, which has many uses as a physiological buffer as its phosphate groups aid in the maintenance of a constant pH of 7.2. It contains the following within distilled water (see Table APP1. b.).

Ion/Reagent	Molecular Weight	Final Concentration
KH_2PO4	136	1.54 mM
NaCl	58	155.17 mM
Na_2HPO_4-7H2O	268	2.71 mM

Table APP1. b. The ingredients for Phosphate-Buffered Saline

APP1. c. Solution A (For Mitochondrial Isolation via Differential Centrifugation see 'M3. Cellular Sub-Fractionation & Mitochondrial Isolation'; page 70)

Solution A is a mitochondrial buffer that is utilised to suspend the fragmented cells during differential centrifugation. The following reagents can be dissolved in distilled water at pH 7.4 to make up 500ml of solution, which should be subsequently stored at 4 ˚C (see Table APP1. c.).

Ion/Reagent	Molecular Weight	Final Concentration
Mannitol	182.17	250 mM
EGTA (Ethylene Glycol (Bis(β-Aminoethylether)-N,N,N,-Tetraacetic Acid	380.4	0.5 mM
HEPES (Organic chemical buffer)	238.3	5 mM

Table APP1. c. The ingredients for Solution A.

APP1. d. Solution B & Percoll (For Mitochondrial Isolation via Differential Centrifugation see 'M3. Cellular Sub-Fractionation & Mitochondrial Isolation'; page 70).

Solution B is a similar mitochondrial buffer that is utilised to suspend the fragmented cells while Percoll density gradient separation occurs. The following reagents can be dissolved in distilled water to make up 100 ml of solution at pH 7.4, which should be subsequently stored at 4 ^{o}C (see Table APP1. d.).

Percoll consists of colloidal silica particles coated with polyvinylpyrrolidone and has a low viscosity and osmolarity with no toxicity, and is therefore well-suited for density gradient separation experiments. When required, Solution B is added to Percoll to make a final solution of 70%/30% (vol/vol) solution.

Ion/Reagent	Molecular Weight	Final Concentration
Mannitol	**182.17**	**225 mM**
EGTA (Ethylene Glycol (Bis(β-Aminoethylether)-N,N,N,-Tetraacetic Acid	**380.4**	**5 mM**
HEPES (Organic chemical buffer)	**238.3**	**25 mM**

Table APP1. d. The ingredients for Solution B.

APP2. Antibodies

APP2. a. Primary Antibodies

Primary antibodies were sourced from a variety of different manufacturers and utilized at manufacturer's recommended concentrations (see Table APP2. a.).

Antibody *RAS Components*	Manufacturer	Catalogue Number	Antibody Type	Molecular Weight	Dilution
Renin (B-12)	**Santa-Cruz Biotechnology** (Dallas, Texas, USA)	**sc-133145**	**Mouse Monoclonal IgG1**	**38-46 kDa**	**1:100**
Renin Antibody	**Atlas Antibodies** (Stockholm, Sweden)	**HPA005131**	**Rabbit Polyclonal IgG**	**40kDa**	**1:250 to 1:500**
ACE (C-20)	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-12187**	**Goat Polyclonal IgG**	**195 kDa**	**1:100**
ACE (N-20)	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-12184**	**Goat Polyclonal IgG**	**195kDa**	**1:200**
ACE (C-Terminal)	**Abcam** (Cambridge, UK)	**ab39172**	**Rabbit Polyclonal IgG**	**170kDa**	**1:1000**
ACE (Clone 9B9)	**Millipore** (Billerica, MA, USA)	**MAB4051**	**Mouse Monoclonal**	**170-180kDa**	**1:100**
Angiotensinogen	**LSBio**	**LS-A9700**	**Rabbit Polyclonal**	**Various**	**1:200**

Antibody	(Seattle, WA, USA)				
Angiotensin (N-10) Antibody	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-7419**	**Goat Polyclonal IgG**	**60kDa**	**1:200**
AT_1R (N-10) Antibody	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-1173**	**Rabbit Polyclonal IgG**	**43kDa**	**1:200**
AT_1R Antibody	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-20255**	**Goat Polyclonal IgG**	**40kDa**	**1:200**
AT_2R	**Abcam** (Cambridge, UK)	**ab19134-50**	**Rabbit Polyclonal**	**44 kDa**	**1:200**
AT_2R (C-18) Antibody	**Santa Cruz Biotechnology** (Dallas, Texas, USA)	**sc-7420**	**Goat Polyclonal IgG**	**44kDa**	**1:200**
Standard Cellular Components					
Cox-4	**Cell Signalling Technology** (Danvers, MA, USA)	**#4844**	**Polyclonal Rabbit**	**17kDa**	**1:1000**
VDAC (Anti-Porin 31HL) Antibody	**Calbiochem** (Merck Group, Darmstadt, Germany)	**529534**	**Mouse IgG**	**20-30kDa**	**1:500**
Grp75	**Santa Cruz**	**sc-1058**	**Goat Polyclonal IgG**	**75 kDa**	**1:200**

	Biotechnology (Dallas, Texas, USA)				
ATP5b	**Abcam** (Cambridge, UK)	**ab37922**	**Chicken Polyclonal**	**57kDa**	**1:2000**
ATP5b [3D5]	**Abcam** (Cambridge, UK)	**ab14730**	**Mouse monoclonal**	**52kDa**	**1:200**
Hoescht 33258	**Life Technologies** (Paisley, UK)	**H3569**	**Nucleic acid stain**	**Binds to DNA**	**1:1000**

Table APP2. a. Primary antibodies utilised for experimentation

APP2. b. Secondary Antibodies

Secondary antibodies were sourced from a variety of different manufacturers and utilized at multiple different concentrations to investigate the optimum working conditions (see Table APP2. b.).

Antibody	Manufacturer	Catalogue Number	Optimal Dilution
Rabbit Anti-Goat IgG (Fc), Peroxidase Conjugated	**Thermo Scientific** (Waltham, MA, USA)	**#31433**	**1:200**
Cy2 Green Conjugated Affinipure Donkey Anti-Chicken	**Jackson Immunoresearch** (West Grove, PA, USA)	**82544**	**1:50**
AlexaFluor® 594 Donkey Anti-Goat IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-11058**	**1:200**
Alexa Fluor® 488 donkey anti-mouse IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-21202**	**1:200**
Alexa Fluor® 488 donkey anti-goat IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-11055**	**1:200**
Alexa Fluor® 594 donkey anti-mouse IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-21203**	**1:200**
Alexa Fluor® 594 donkey anti-rabbit IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-21207**	**1:200**

Alexa Fluor® 647 donkey anti-mouse IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-31571**	**1:200**
Alexa Fluor® 647 donkey anti-goat IgG	**Invitrogen** (Carlsbad, CA, USA)	**A-21447**	**1:200**

Table APP2. b. Secondary antibodies utilized for experimentation

APP3. Complete Mass Spectrometry Results

	Description	mW (Da)	PLGS Score	Coverage (%)
Q6IG03	K2C73 RAT Keratin type II cytoskeletal 73 OS Rattus norvegicus GN Krt73 PE 1 SV 1	60349	284.036	36.528
Q6IMF3	K2C1 RAT Keratin type II cytoskeletal 1 OS Rattus norvegicus GN Krt1 PE 2 SV 1	64790	505.1323	29.44
Q68FR8	TBA3 RAT Tubulin alpha 3 chain OS Rattus norvegicus GN Tuba3a PE 2 SV 1	49927	497.5478	40.4444
Q4FZU2	K2C6A RAT Keratin type II cytoskeletal 6A OS Rattus norvegicus GN Krt6a PE 1 SV 1	59212	211.974	33.8768
Q6IG01	K2C1B RAT Keratin type II cytoskeletal 1b OS Rattus norvegicus GN Krt77 PE 2 SV 1	57219	252.1117	27.9383
P11980	KPYM RAT Pyruvate kinase isozymes M1 M2 OS Rattus norvegicus GN Pkm2 PE 1 SV 3	57780	179.5759	28.4369
P00924	ENO1 YEAST Enolase 1 OS Saccharomyces cerevisiae GN ENO1 PE 1 SV 2	46773	297.3125	37.2998
Q6AY56	TBA8 RAT Tubulin alpha 8 chain OS Rattus norvegicus GN Tuba8 PE 2 SV 1	50005	318.9343	23.3853
P07323	ENOG RAT Gamma enolase OS Rattus norvegicus GN Eno2 PE 1 SV 2	47110	296.8302	29.4931
P85108	TBB2A RAT Tubulin beta 2A chain OS Rattus norvegicus GN Tubb2a PE 1 SV 1	49874	256.935	33.2584
Q6P9T8	TBB2C RAT Tubulin beta 2C chain OS Rattus norvegicus GN Tubb2c PE 1 SV 1	49769	244.966	25.618
Q6AYZ1	TBA1C RAT Tubulin alpha 1C chain OS Rattus norvegicus GN Tuba1c PE 1 SV 1	49905	373.2762	26.9488
Q6P9V9	TBA1B RAT Tubulin alpha 1B chain OS Rattus norvegicus GN Tuba1b PE 1 SV 1	50119	429.9057	21.286
P04764	ENOA RAT Alpha enolase OS Rattus norvegicus GN Eno1 PE 1 SV 4	47098	322.2678	19.1244
P69897	TBB5 RAT Tubulin beta 5 chain OS Rattus norvegicus GN Tubb5 PE 1 SV 1	49638	293.6011	21.1712
P15429	ENOB RAT Beta enolase OS Rattus norvegicus GN Eno3 PE 1 SV 3	46984	272.7215	21.659
Q3KRE8	TBB2B RAT Tubulin beta 2B chain OS Rattus norvegicus GN Tubb2b PE 1 SV 1	49920	247.7995	11.4607

Table APP3. a. A table of database search results, following mass spectrometry performed at University College London, demonstrated no findings consistent with ACE, but multiple contaminants (keratin, dermicidin) and experimental reagents (trypsin, enolase) (mW- Molecular weight; Da- Daltons; PLGS Score- Protein Lynx Global Server Score).

Gel Piece	Accession	Description	mW (Da)	PLGS Score	Coverage (%)
1	P70610	DOC2B RAT Double C2 like domain containing protein beta OS Rattus norvegicus GN Doc2b PE 1 SV 2	45812	76.1767	2.1845
2	P70610	DOC2B RAT Double C2 like domain containing protein beta OS Rattus norvegicus GN Doc2b PE 1 SV 2	45812	63.0816	2.1845

Table APP3.2. Two gel pieces analysed at University College London demonstrated no significant correlations and no findings consistent with ACE (mW- Molecular weight; Da- Daltons; PLGS Score- Protein Lynx Global Server Score).

Description	dCn	Ions	XCorr
URIC_RAT Uricase OS=Rattus norvegicus GN=Uox PE=1 SV=3	0.3522	16/20	3.1180
ACTA_RAT Actin, aortic smooth muscle OS=Rattus norvegicus GN=Acta2 PE=1 SV=1	0.1048	28/60	4.0710
ANXA1_RAT Annexin A1 OS=Rattus norvegicus GN=Anxa1 PE=1 SV=2	0.3589	21/26	5.0531
ACTB_RAT Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1	0.2091	27/68	4.1511
ANXA2_RAT Annexin A2 OS=Rattus norvegicus GN=Anxa2 PE=1 SV=2	0.2074	35/68	5.2817
RLA0_RAT 60S acidic ribosomal protein P0 OS=Rattus norvegicus GN=Rplp0 PE=1 SV=2	0.4936	20/32	5.1187
1433Z_RAT 14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1	0.3792	22/26	5.0860
ENOA_RAT Alpha-enolase OS=Rattus norvegicus GN=Eno1 PE=1 SV=4	0.5344	22/34	4.5639

Table APP3.3. A table of database search results, following mass spectrometry performed at Harvard Medical School demonstrated no findings consistent with ACE (dCn- Delta correlation score; XCorr- Cross correlation score).